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PRINCIPAL INVESTIGATOR: Duen-Hwa Yan, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

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Duen-Hwa Yan, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-Mail: dyan@mdanderson.org

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13. ABSTRACT (Maximum 200 Words)

In this project, we attempt to establish the utility of an antisense iron-responsive element (AS-IRE)-mediated gene expression system to targeting HER-2/neu-overexpressing breast cancer cells. During the funding period, we have finished the proposed goal stated in Task 1 by identifying the "optimal" HER-2/neu antisense IRE, i.e., AS-IRE4. Moreover, we showed preferential cell killing in HER-2/neu-overexpressing MDA-MB-453 cells using hTERT-AS-IRE4-Bax as opposed to in low HER-2/neu-expressing MDA-MB-468 cells (Task 2). The results obtained from Task 1 and 2 have been published in Cancer Letters (174:151-158, 2001). To test the therapeutic efficacy of the hTERT-AS-IRE4-luc in a pre-clinical gene therapy model (Task 3), our preliminary results shows hTERT-AS-IRE4-Bax can be used in vivo as a therapeutic agent in non-HER-2 overexpressing breast tumors if the iron level is deregulated in these tumors due to TfR overexpression. Since HER-2/neu overexpression tumors comprise only about 20-30% of breast cancer, our new data may broaden the application of AS-IRE strategy in breast cancer treatment. Our next goal is to use a binary adenoviral vector Bax gene expression system to test the preferential killing of HER-2-overexpressing breast cancer cells in vitro and in vivo. We showed that adenoviral vector-mediated therapeutic gene transfer could yield treatment efficacy. In addition, we have explored the possibility of using small interference RNA (SiRNA) as a novel approach to specific target HER-2-overexpressing cells. Our preliminary results suggested that SiRNA is potentially a powerful technology to achieve specific downregulation of HER-2/neu gene.

14. SUBJECT TERMS
Breast cancer, HER-2/neu overexpression, iron-responsive element, antisense, pre-clinical gene therapy

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INTRODUCTION

The overexpression of HER-2/neu proto-oncogene has been found in a variety of human cancers. In particular, amplification and overexpression of the HER-2/neu gene were found in 20-30% of patients with breast cancer. These patients had decreased survival and increased relapse rates. Therefore, HER-2/neu overexpression has been used as a poor prognostic indicator for patients suffering from this disease. To develop an expression system that targets the breast cancer cells overexpressing HER-2/neu mRNA, a novel approach is described that combines the antisense principle and the biochemical property of a translation regulator, an iron-responsive element (IRE). IRE, when placed 5' to a gene, functions as a negative translation regulator in that IRE interacts with iron-regulatory proteins (IRPs) and this protein-RNA complex blocks translation (1). One way to alleviate this translation inhibition is to prevent the IRE/IRP interaction by disrupting the IRE stem-loop structure via a sense-antisense hybrid. Thus, a HER-2/neu antisense IRE (AS-IRE) possessing the IRE consensus sequence and functioning as a translation inhibitor was generated. When placed 5' to a reporter gene, AS-IRE could direct the reporter gene expression in breast cancer cells that overexpress HER-2/neu mRNA, since the IRE-mediated translation inhibition can be overcome by the overexpression of HER-2/neu mRNA. In this project, we attempt to establish the utility of this novel approach to targeting HER-2/neu-overexpressing breast cancer cells. Our goals are: (1) to obtain an optimal AS-IRE that directs the maximum expression of the reporter gene in HER-2/neu overexpressing breast cancer cells; (2) to demonstrate a preferential killing of HER-2/neu overexpressing breast cancer cells by using the optimal HER-2/neu antisense IRE to direct the expression of a toxin gene encoding diphtheria toxin A-chain (DT-A); and (3) to test the therapeutic effect of the AS-IRE-mediated DT-A expression vector *in vivo* by treating the mice that bear tumors with or without the overexpression of HER-2/neu gene.

BODY

(Figures are attached in the Appendices)

Task 1: To obtain an optimal HER-2/neu antisense IRE.

We identified AS-IRE4 as the "optimal" AS-IRE in that AS-IRE4 behaves as a canonical IRE by interacting with IRP-1 and regulated by iron. Importantly, AS-IRE4 is able to direct a preferential gene expression in HER-2/neu-overexpressing breast cancer cells (2).

Task 2: To obtain an optimal HER-2/neu antisense IRE-regulated toxin gene (DT-A).

Using Bax (a pro-apoptotic protein in the Bcl-2 protein family (3)), as the therapeutic gene, directed by AS-IRE4 and a tumor-specific promoter of hTERT gene, we showed that hTERT-AS-IRE4-Bax could be used to achieve a preferential cell killing in breast cancer cells that overexpress HER-2/neu mRNA (2).

Task 3: To demonstrate the therapeutic effect of the HER-2/neu antisense IRE-mediated gene expression.

With a successful demonstration of preferential killing in HER-2/neu-overexpressing cells shown above (see **Task 2**), we tested the therapeutic efficacy of hTERT-AS-IRE4-Bax gene therapy treatment in an orthotopic breast cancer xenograft model. To achieve tumor-specific killing, we combined AS-IRE strategy with a human telomerase reverse transcriptase (hTERT) gene promoter (that is highly active in tumor cells but is repressed in normal primary cells) to direct the gene expression of a pro-apoptotic gene, Bax. Since few HER-2/neu overexpressing breast cancer cell lines are tumorigenic, the one is tumorigenic often grows very slowly in mice, e.g., MDA-MB-361. To test the efficacy of hTERT-AS-IRE4-Bax *in vivo*, we used MDA-MB-231 as a model for two reasons. One, AS-IRE4 functions as a typical IRE that can be regulated by iron (2), and two, MDA-MB-231 overexpresses transferrin receptor (TfR) leading to increased cellular iron concentration that has been associated with metastatic breast cancer (4). The active hTERT and the high cellular iron level in MDA-MB-231 cells will allow the Bax gene to be expressed resulting in tumor killing. To test this hypothesis, we treated the mice that bore MDA-MB-231 tumors either with hTERT-AS-IRE4-Bax or the control, hTERT-AS-IRE4-luc using SN2 liposome as gene delivery system. As shown in Figure 1, a significant anti-tumor activity is seen in mice treated with hTERT-AS-IRE4-Bax as compared with that treated with hTERT-AS-IRE4-luc. This preliminary result shows, at least in principle, hTERT-AS-IRE4-Bax can be used *in vivo* as a therapeutic agent in non-HER-2 overexpressing breast tumors if the iron level is deregulated in these tumors due to TfR overexpression. Since HER-2/neu overexpression tumors comprise only about 20-30% of breast cancer, our new data may broaden the application of AS-IRE strategy in breast cancer treatment.

We will use a binary adenoviral vector Bax gene expression system to test the preferential killing of HER-2-overexpressing breast cancer cells *in vitro* and *in vivo*. This viral gene expression system (5), developed by our collaborator, Dr. Bingliang Fang, Department of Thoracic and Cardiovascular Surgery, MDACC, consists of two adenoviruses (Ad): first, Ad-GT-Bax, it carries Bax gene controlled by a synthetic GAL4-responsive promoter (GT) that contains five GAL4-binding sites and a TATA box. GT is silent in 293 packaging cells and thus avoiding the toxic effects of Bax expression during virus production. Second, Ad-hTERT-GV16, it carries GAL4/VP16 fusion protein (GV16) that activates GT-Bax in tumors (but not in normal tissues) resulting in Bax expression and cell killing. We tested the binary adenovirus system and showed the expression of Bax in breast cancer cells infected with Ad-GT-Bax and Ad-hTERT-GV16 (data not shown). We will construct Ad-hTERT-GV16 and Ad-GT-AS-IRE-Bax (AS-IRE-Bax under the control of GT promoter). If our hypothesis is correct, we expect to see preferential killing of the HER-2-overexpressing breast cancer cells infected with Ad-hTERT-GV16 and Ad-GT-AS-IRE-Bax (Fig. 2).

To demonstrate the feasibility of using adenoviral vector-mediated gene transfer system in pre-clinical gene therapy for breast cancer, we constructed a recombinant adenoviral vector carrying a therapeutic gene, p202 (Ad-p202) (6). We showed that Ad-p202 treatment via intra-tumor (Fig. 3A) or systemic intra-venous (Fig. 3B) injection resulted in significant anti-tumor effect on an orthotopic breast cancer xenograft model derived from human breast cancer cell line MDA-MB-468. These results suggest that the use of adenovirus-based gene therapy is feasible in our experimental gene therapy system.

During the past year, we also explored other novel approach to target HER-2-overexpressing breast cancer cells, e.g., by downregulating HER-2 using RNA interference (RNAi) (7, 8). To test this idea, we generated small interference RNAs (SiRNA) that are specifically targeting HER-2 mRNA (Si-HER2) or the control luciferase mRNA (Si-GL3) (Fig. 4A). To test the specificity of S-RNA-mediated gene silencing, we performed a reporter assay in which a luciferase reporter gene driven by a TERT promoter (TERT-GL3-luc) was co-transfected with either Si-GL3 or Si-HER2 into 293 cells. As shown in Fig. 4B, we showed that Si-GL3 transfection completely abolished luciferase gene expression but not Si-HER2 or the TERT-GL3-luc alone (buffer). This observation confirmed the specific gene silencing effect by SiRNAs. To demonstrate the HER-2 specific gene silencing by Si-HER2, we co-transfected a HER-2 expression vector driven by a CMV promoter (CMV-HER2) with either Si-HER2 or the control Si-GL3 into 293 cells. Forty-eight hours post-transfection, cell lysates were isolated and analyzed for HER-2 protein expression by western blot. We showed that HER-2 protein expression is specifically downregulated in Si-HER2 transfected cells but not in those transfected with Si-GL3 or HER-2 expression vector alone (buffer) (Fig. 4C). These results clearly show th feasibility of using SiRNA-based treatment to target HER-2-overexpressing cells.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of AS-IRE4 as the "optimal" AS-IRE.
- Construction of hTERT-AS-IRE4-luc.
- Demonstration of iron-regulated gene expression using hTERT-AS-IRE4-luc.
- Demonstration of HER-2/neu overexpression-specific expression using hTERT-AS-IRE4-luc.
- Demonstration of HER-2/neu overexpression-specific cell killing using hTERT-AS-IRE4-Bax.
- Demonstration of the feasibility of using a liposome-based gene transfer system to deliver hTERT-AS-IRE4-Bax and to achieve treatment efficacy in a breast cancer xenograft model.
- Demonstration of the feasibility of using an adenovirus-based gene transfer system to achieve treatment efficacy in a breast cancer xenograft model.
- Demonstration of the feasibility of using SiRNA technology to specifically target HER-2-overexpressing cells.

REPORTABLE OUTCOMES

Publications:

- Li, Z., Xia, W., Fang, B., and Yan, D.-H. (2001) Targeted gene expression in HER-2/neu-overexpressing breast and ovarian cancer cells by an antisense iron responsive element. *Cancer Lett.* 174:151-158

- Ding, Y., Wen, Y., Spohn, B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Zheng, L., Hortobagyi, G. N., Hung, M.-C., and Yan, D.-H. (2002) Pro-apoptotic and anti-tumor activities of adenovirus-mediated p202 gene transfer. *Clin. Cancer Res.* 8:3290-3297.

- Yan, D.-H., Abramian, A., Li, Z., Ding, Y., Wen, Y., Liu, T.-J., and Hunt, K. (2003) p202, an interferon-inducible protein, inhibits E2F1-mediated apoptosis in prostate cancer cells. *Biochem. Biophys. Res. Comm.* 303:219-222.

Meeting abstracts:

- Li, Z.* and Yan, D.-H. Targeted gene expression in HER-2/neu-overexpressing breast and ovarian cancer cells by an antisense iron responsive element. (Poster presentation) American Association for Cancer Research, 92nd annual meeting, March 24-28, 2001, New Orleans, LA. *Recipient of a post-doctoral travel award from AACR

- Yan, D.-H, Wen, Y., Ding, Y., Spohn, B., Wang, B., Shao, R., Zou, Y., Xie, K., Hortobagyi, G. N., and Hung, M.-C. Anti-tumor Activities of p202. (Oral presentation) 6th World Congress on Advances in Oncology and 4th International symposium on Molecular Medicine, October 18-20, 2001, Hersonissos, Crete, Greece.

- Yan, D.-H., Ding, Y., Wen, Y., Spohn, B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., and Hung, M.-C. Pro-apoptotic and anti-tumor activities of adenovirus-mediated p202 gene transfer. (Poster presentation) American Association for Cancer Research, 93rd annual meeting, April 6-10, 2002, San Francisco, CA.

- Li, Z., Xia, W., Fang, B., and Yan, D.-H. Targeting HER-2/neu-overexpressing breast cancer cells by an antisense iron-responsive element-directed Bax gene expression. (Poster presentation) Era of Hope, September 25-28, 2002, Orlando, Florida.

Personnel receiving pay from the research effort

Zheng Li, post-doctoral fellow

CONCLUSIONS

We have finished the proposed goal stated in **Task 1** and **Task 2**. The results have been published in ***Cancer Letters*** (174:151-158, 2001). To test the therapeutic efficacy of the hTERT-AS-IRE4-luc in a pre-clinical gene therapy model (**Task 3**), our preliminary result shows hTERT-AS-IRE4-Bax can be used *in vivo* as a therapeutic agent in non-HER-2 overexpressing breast tumors if the iron level is deregulated in these tumors due to TfR overexpression. Since HER-2/neu overexpression tumors comprise only about 20-30% of breast cancer, our new data may broaden the application of AS-IRE strategy in breast cancer treatment. Our next goal is to use a binary adenoviral vector Bax gene expression system to test the preferential killing of HER-2-overexpressing breast cancer cells *in vitro* and *in vivo*. We showed that adenoviral vector-mediated gene transfer system could yield therapeutic efficacy in our breast cancer orthotopic xenograft model. In addition, we demonstrated the feasibility of using SiRNA as a novel approach to specific target HER-2-overexpressing cells.

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APPENDICES

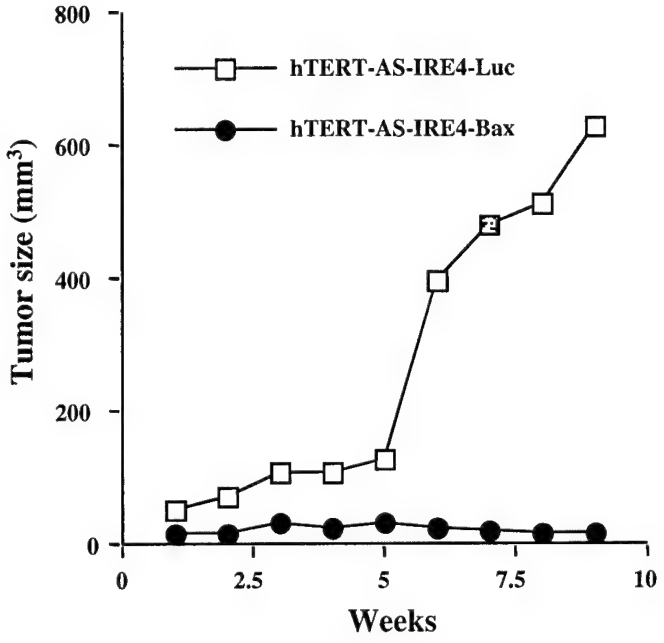

Fig. 1. Treatment efficacy by hTERT-AS-IRE4-Bax in MDA-MB-231 xenograft model. MDA-MB-231 cells (2 x 10^6) were implanted into mammary fat pads of female nude mice. 15 μg of DNA (hTERT-AS-IRE4-Bax or hTERT-AS-IRE4-luc) and 15 μl of SN2 liposome complex were used for each treatment. Treatment began when tumor size reached 5 mm in diameter by intra-tumor injection twice per week.

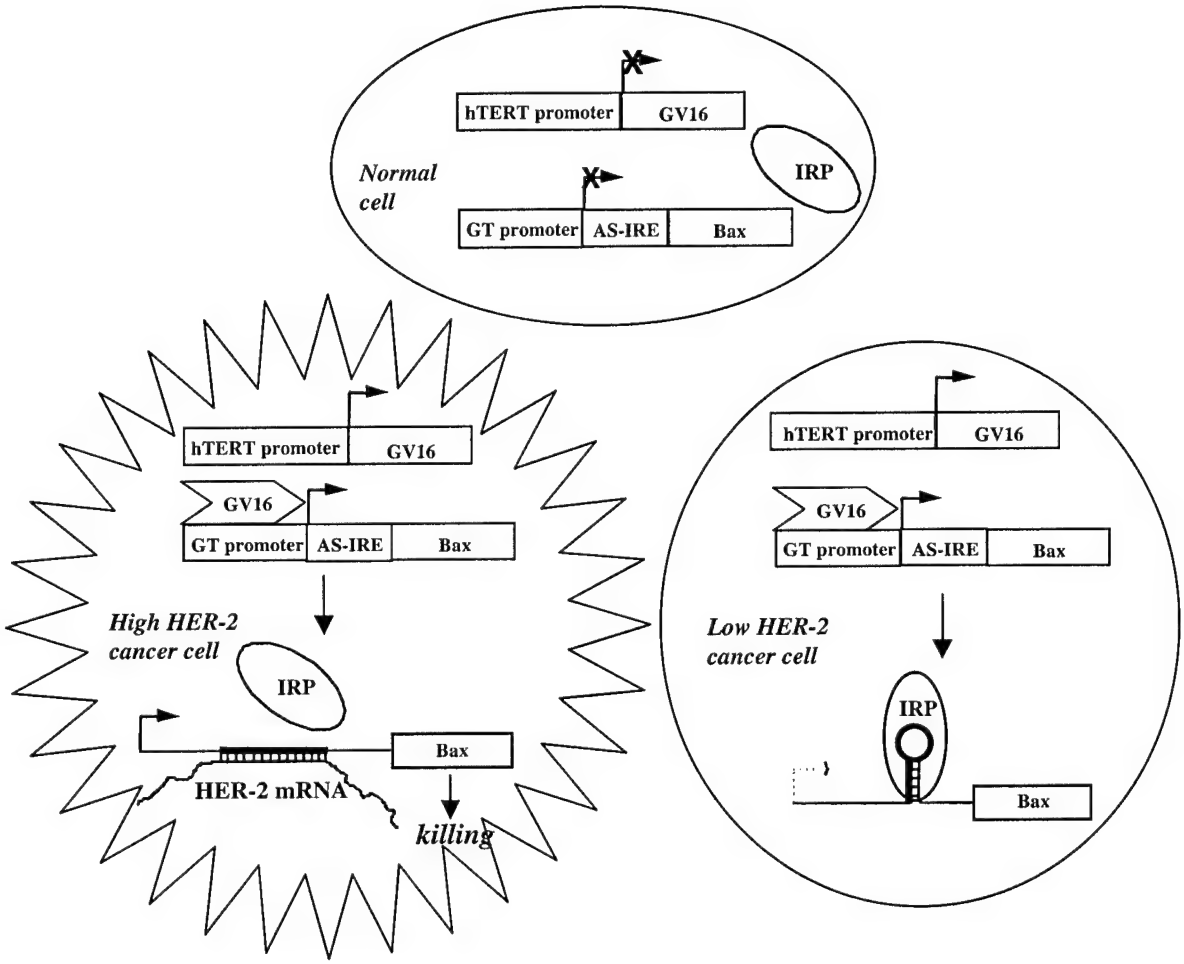

Fig. 2. Specific targeting the HER-2-overexpressing cancer cell by Ad-GT-AS-IRE-Bax/Ad-hTERT-GV16 binary adenoviral vector system. Co-infecting Ad-GT-AS-IRE-Bax and Ad-hTERT-GV16 into normal cell yields no cell killing due to the lack of GV16 expression because the hTERT promoter is inactive. In contrast, hTERT promoter is active in cancer cell and thus allows GV16 expression to activate GT promoter to transcribe AS-IRE4-Bax mRNA. However, AS-IRE4-Bax mRNA is not translated due to AS-IRE/IRP interaction in low HER-2 cancer cell. Such translational inhibition is alleviated in High HER-2 cell since HER-2 mRNA forms sense/antisense hybrid with AS-IRE4, and thus allows Bax expression leading to cell killing.

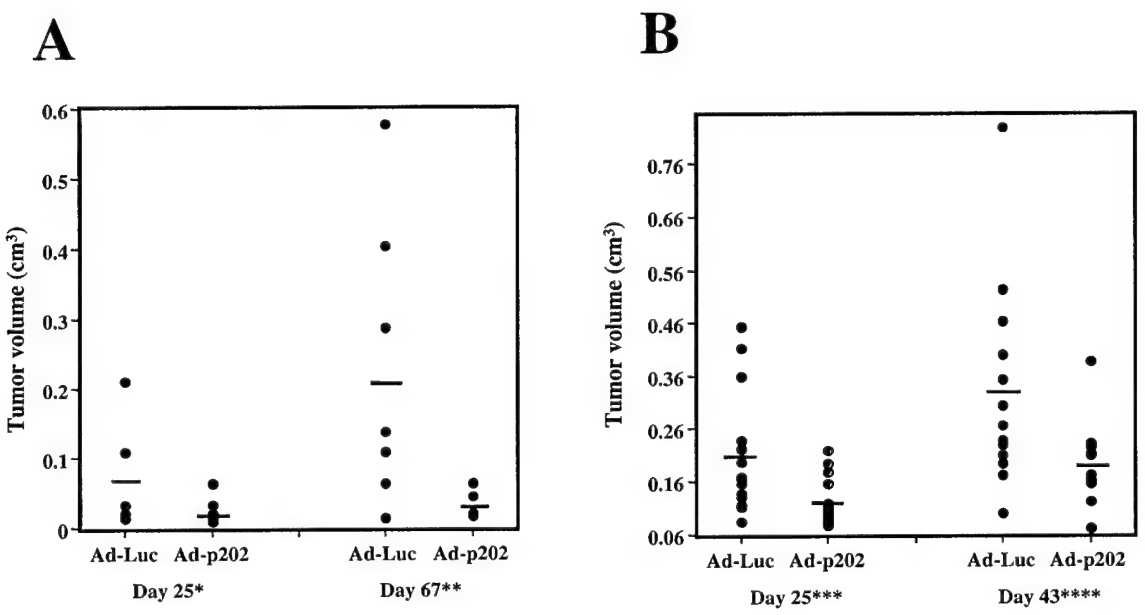

Fig. 3. Anti-tumor effect by systemic delivery of Ad-p202 on an orthotopic breast cancer xenograft model. *A*. Ad-p202-mediated anti-tumor effect on breast cancer xenografts by intra-tumor treatment. MDA-MB-468 cells (2 x 10^6 cells) were implanted in mammary fat pads of each female nude mouse. Tumor-bearing mice were divided into two treatment groups: Ad-Luc (total 7 tumors) and Ad-p202 (total 7 tumors), at 1 x 10^9 pfu per treatment via intra-tumor injection. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for seven weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, i.e., day 25 and day 67. *t*-test: * $p = 0.13$ and ** $p = 0.04$. *B*. Ad-p202-mediated anti-tumor effect on breast cancer xenografts by systemic treatment. MDA-MB-468 cells (2 x 10^6 cells) were implanted in mammary fat pads (2 tumors per mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups: Ad-Luc (Luc) (total 14 tumors) and Ad-p202 (p202) (total 14 tumors), at 5 x 10^8 pfu via tail vein injection. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for five weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, i.e., day 25 and day 43. *t*-test: *** $p = 0.0097$ and **** $p = 0.014$. *C*. Apoptosis correlates with p202 expression in Ad-p202-treated breast tumors. Mice were sacrificed 24 h after the last systemic treatment as described above. Tumors were then excised and fixed for the subsequent immunohistochemical analysis. p202 expression was analyzed by using antibody specific for p202 on tumor samples obtained from Ad-p202 or Ad-Luc treated mice (14). TUNEL assay was also performed to detect apoptotic cells in these tumors (15). The arrows indicate the representatives of apoptotic cells.

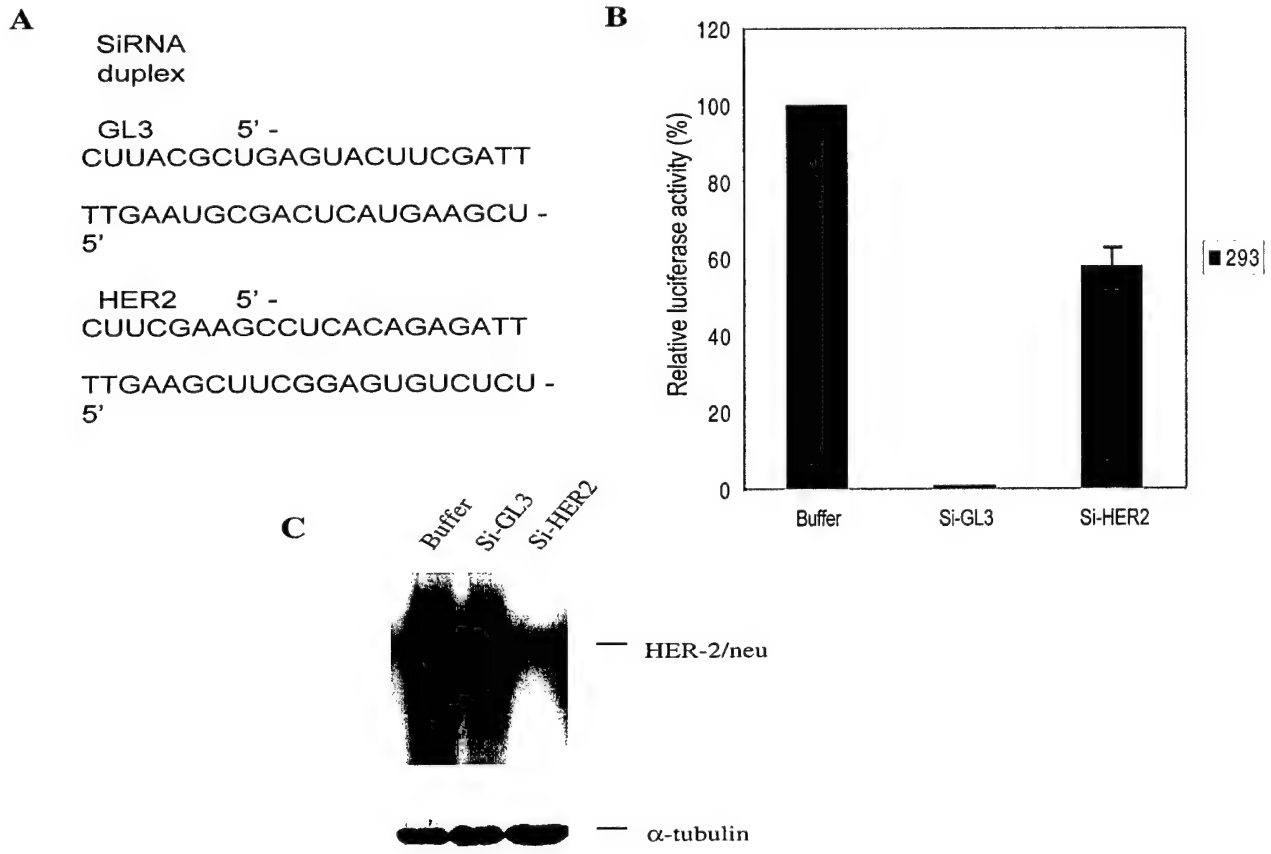

Fig. 4. HER-2 gene silencing by specific SiRNA. A. The sense (top) and antisense (bottom) sequences of the SiRNA duplexes specifically targeting GL3 luciferase (Si-GL3) and HER-2 (Si-HER2) mRNAs used in the experiment. B. Specific luciferase gene silencing by Si-GL3. TERT-GL3-luc (2 μg), pRL-TK (0.1 μg) plasmid (Promega) and Si-HER2 or Si-GL3 (0.25 μg) were co-transfected into 293 cells. The luciferase activity was measured 20-h post-transfection using the Dual luciferase reporter assay kit (Promega). C. Specific HER-2 downregulation by Si-HER2. CMV-HER2 (3 μg) and Si-HER2 or Si-GL3 (0.25 μg) were co-transfected into 293 cells. The cell lysates were prepared 20-h post-transfection. Western blot analysis was performed using antibody against HER-2. The same membrane was then stripped and re-probed with α–tubulin antibody to serve as loading control.

Meeting abstract

Li, Z.* and Yan, D.-H. Targeted gene expression in HER-2/neu-overexpressing breast and ovarian cancer cells by an antisense iron responsive element. (Poster presentation) American Association for Cancer Research, 92nd annual meeting, March 24-28, 2001, New Orleans, LA. *Recipient of a post-doctoral travel award from AACR

Overexpression of HER-2/neu proto-oncogene in tumor is found in 20-30% breast or ovarian cancer patients and is a predictor of poor prognosis. To target HER-2/neu overexpressing cancer cells, we have previously described a novel strategy that combines the antisense principle and translational inhibition conferred by an iron responsive element (IRE) (Biochem. Biophys.Res. Commun. 246:353-358). In that, a HER-2/neu antisense IRE (AS-IRE), when placed in the 5'UTR of a reporter gene, inhibits translation. This translational inhibition could be preferentially alleviated in HER-2/neu-overexpressing breast cancer cells, presumably by the formation of HER-2/neu sense/antisense hybrid that abolishes AS-IRE and its interaction with iron regulatory proteins (IRPs). In this study, 12 potential AS-IREs were screened for their ability to preferentially express a reporter gene in HER-2/neu overexpressing cancer cells. Using RNA retardation assay, we showed that all the AS-IREs, rather than a mutant IRE, could compete the IRPs binding to the IRE derived from the 3'UTR of transferrin receptor mRNA, suggesting that AS-IREs could specifically bind IRPs. We found that one of the AS-IREs, AS-IRE4, exerts the highest sensitivity to IRP-mediated translational inhibition. Importantly, in addition to HER-2/neu-overexpressing breast cancer cells, AS-IRE4 could direct preferential gene expression in ovarian cancer cells that overexpress HER2/neu gene. Further modification on AS-IRE4, we obtained the AS-IRE4-M which could further improve the preferential gene expression in HER-2/neu-overexpressing breast and ovarian cancer cells. Our results suggest a potential application of using AS-IRE strategy to target HER-2/neu-overexpressing cancer cells.

Meeting abstract

Yan, D.-H, Wen, Y., Ding, Y., Spohn, B., Wang, B., Shao, R., Zou, Y., Xie, K., Hortobagyi, G. N., and Hung, M.-C. Anti-tumor Activities of p202. (Oral presentation) 6th World Congress on Advances in Oncology and 4th International symposium on Molecular Medicine, October 18-20, 2001, Hersonissos, Crete, Greece.

p202, an interferon (IFN)-inducible protein, is a member of murine 200-amino acid repeat family. Ectopic expression of p202 in human prostate, breast, and pancreatic cancer cells resulted in multiple anti-tumor activities that include growth inhibition, sensitization to TNF-α-induced apoptosis, suppression of metastasis and angiogenesis. Using adenovirus-mediated gene delivery system to express p202 gene (Ad-p202) in breast cancer cells, we also demonstrated that Ad-p202 infection induces growth inhibition as well as sensitizes the otherwise resistant cells to TNF-α-induced apoptosis. Importantly, we showed for the first time that Ad-p202 infection alone induces apoptosis, and that requires the activation of caspases (caspase-3, in particular) for full apoptotic effect. Together, our results suggest that Ad-p202 is a potent growth-suppressing, pro-apoptotic agent that not only provides a useful tool to investigate the function of p202, but also can be used to further study the treatment efficacy in breast cancer xenograft model.

Meeting abstract

Yan, D.-H., Ding, Y., Wen, Y., Spohn, B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., and Hung, M.-C. Pro-apoptotic and anti-tumor activities of adenovirus-mediated p202 gene transfer. (Poster presentation) American Association for Cancer Research, 93rd annual meeting, April 6-10, 2002, San Francisco, CA.

Purpose and Experimental Design:

p202, an interferon-inducible protein, is a member of the murine 200-amino acid repeat family. Enforced p202 expression in stable cancer cell lines resulted in growth inhibition *in vitro* and tumor suppression *in vivo*. However, to study the immediate effect of p202 and to test the potential efficacy of p202 treatment, an efficient gene delivery system for p202 is required. For these purposes, an adenoviral vector expressing the p202 gene (Ad-p202) was generated. We examined the effects of Ad-p202 infection on human breast cancer cells. Furthermore, we tested the efficacy of Ad-p202 treatment on breast and pancreatic cancer xenograft models.

Results:

We found that Ad-p202 infection induces growth inhibition and sensitizes the otherwise resistant cells to TNF-α-induced apoptosis. In addition, we demonstrated for the first time that Ad-p202 infection induces apoptosis, and that requires the activation of caspase-3 for full apoptotic effect. More importantly, we showed the efficacy of Ad-p202 treatment on both breast and pancreatic cancer xenograft models, and this anti-tumor effect correlated well with enhanced apoptosis in Ad-p202-treated tumors.

Conclusions:

We conclude that Ad-p202 is a potent growth inhibitory, pro-apoptotic and tumor-suppressing agent. Ad-p202 may be further developed into an efficient therapeutic agent for human cancer gene therapy.

Meeting abstract

Li, Z., Xia, W., Fang, B., and Yan, D.-H. Targeting HER-2/neu-overexpressing breast cancer cells by an antisense iron-responsive element-directed Bax gene expression. (Poster presentation) Era of Hope, September 25-28, 2002, Orlando, Florida.

Overexpression of HER-2/neu proto-oncogene is found in many human cancers including 20-30% of breast cancer and is a predictor of poor prognosis. To target breast cancer cells that overexpress HER-2/neu mRNA, we previously described a novel strategy that combines the principle of antisense (AS) and translational inhibitory activity conferred by an iron-responsive element (IRE) (AS-IRE). Here, we showed that three potential AS-IREs, i.e., AS-IRE1, 4, and 5, derived from HER-2/neu antisense sequence could bind endogenous iron regulatory protein (IRP) and, when placed in 5' untranslated region (5'UTR) of a reporter gene, the gene expression could be translationally repressed by recombinant IRP in vitro. Using AS-IRE4 as our model, we demonstrated that it is regulated by iron, and importantly, such regulation is impaired in HER-2/neu-overexpressing breast cancer cells. Furthermore, we showed that AS-IRE4 could preferentially direct the expression of a reporter gene in HER-2/neu-overexpressing breast cancer cells. Interestingly, when AS-IRE4 was placed in 5'UTR of Bax gene, a pro-apoptotic protein in the Bcl-2 protein family, we observed a preferential cell killing in breast cancer cells that overexpress HER-2/neu. Taken together, our results suggest that AS-IRE behaves as a functional IRE and it may direct therapeutic gene expression to preferentially target HER-2/neu-overexpressing breast cancer cells.

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Targeting HER-2/neu-overexpressing breast cancer cells by an antisense iron responsive element-directed gene expression

Zheng Li[a], Weiya Xia[a], Bingliang Fang[b], Duen-Hwa Yan[a,c,*]

[a]*Department of Molecular and Cellular Oncology, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd. Houston, TX 77030, USA*

[b]*Department of Thoracic and Cardiovascular Surgery, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd. Houston, TX 77030, USA*

[c]*Department of Surgical Oncology, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd. Houston, TX 77030, USA*

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Targeting HER-2/neu-overexpressing breast cancer cells by an antisense iron responsive element-directed gene expression

Zheng Li[a], Weiya Xia[a], Bingliang Fang[b], Duen-Hwa Yan[a,c,*]

[a]*Department of Molecular and Cellular Oncology, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd. Houston, TX 77030, USA*

[b]*Department of Thoracic and Cardiovascular Surgery, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd. Houston, TX 77030, USA*

[c]*Department of Surgical Oncology, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd. Houston, TX 77030, USA*

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Abstract

Overexpression of HER-2/neu proto-oncogene is found in many human cancers including 20–30% of breast cancer and is a predictor of poor prognosis. To target breast cancer cells that overexpress HER-2/neu mRNA, we previously described a novel strategy that combines the principle of antisense (AS) and translational inhibitory activity conferred by an iron-responsive element (IRE) (AS-IRE). Here, we showed that three potential AS-IREs, i.e. AS-IRE1, 4, and 5, derived from HER-2/neu antisense sequence could bind endogenous iron regulatory protein (IRP) and, when placed in 5′ untranslated region (5′UTR) of a reporter gene, the gene expression could be translationally repressed by recombinant IRP in vitro. Using AS-IRE4 as our model, we demonstrated that it is regulated by iron, and importantly, such regulation is impaired in HER-2/neu-overexpressing breast cancer cells. Furthermore, we showed that AS-IRE4 could preferentially direct the expression of a reporter gene in HER-2/neu-overexpressing breast cancer cells. Interestingly, when AS-IRE4 was placed in 5′UTR of Bax gene, a pro-apoptotic protein in the Bcl-2 protein family, we observed a preferential cell killing in breast cancer cells that overexpress HER-2/neu. Taken together, our results suggest that AS-IRE behaves as a functional IRE and it may direct therapeutic gene expression to preferentially target HER-2/neu-overexpressing breast cancer cells.

Keywords: Antisense; Iron-responsive element; HER-2/neu; Bax; Gene expression

1. Introduction

The overexpression of HER-2/neu proto-oncogene has been described in a variety of human cancers [1–3]. In particular, amplification and overexpression of the HER-2/neu gene is found in 20–30% of patients with breast or ovarian cancer [4,5] and is associated with an increased relapse rate and decreased survival. Although numerous approaches have been employed to treat HER-2/neu-overexpressing tumors [2,6], novel strategies that offer a specific therapeutic effect on these tumors are clearly needed.

To develop a gene expression system that preferentially targets the breast cancer cells that overexpress HER-2/neu mRNA, we previously described a novel approach that combines the antisense principle and the biochemical properties of a translational regulator, IRE [7] (Fig. 1). IRE, when placed in the 5′UTR of a gene, functions as a negative translational regulator by interacting with IRP and forming an IRE/IRP

* Corresponding author. Tel.: +1-713-792-3677; fax: +1-713-794-0209.

E-mail address: dyan@notes.mdacc.tmc.edu (D.-H. Yan).

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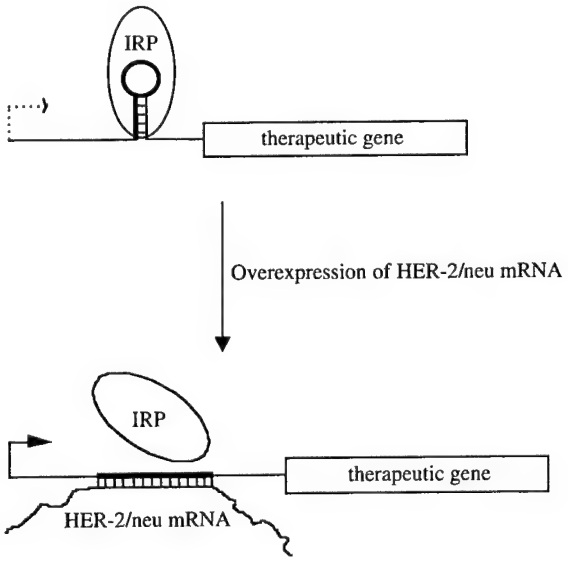

Fig. 1. The AS-IRE-mediated gene expression to target HER-2/neu overexpressing cells. The binding of IRP to AS-IRE (the thick line represents HER-2/neu antisense) results in an inhibition of translation of a therapeutic gene mRNA. The translational inhibition could be overcome in the presence of overexpressed HER-2/neu mRNA by the formation of a sense/antisense hybrid. Specifically, the abolishment of AS-IRE would alleviate the IRP/AS-IRE interaction and leads to restoration of the therapeutic gene expression in HER-2/neu overexpressing cells.

complex that blocks translation [8–10]. One way to alleviate this translational block is to prevent the IRE/IRP interaction by disrupting the IRE stem-loop structure via a sense-antisense hybrid [7]. Based on this idea, we incorporated a HER-2/neu antisense sequence into an IRE to generate AS-IRE that could function as a translational inhibitor when placed in the 5′UTR of a reporter gene. Our model predicts that the IRP/AS-IRE-mediated translational inhibition could be overcome in the presence of overexpressed HER-2/neu mRNA due to the formation of a sense/antisense hybrid. The abolishment of AS-IRE would alleviate the translational inhibition and, in turn, lead to expression of a gene of interest, e.g. a therapeutic gene (Fig. 1). Although AS-IRE preferentially directed the expression of a reporter gene in HER-2/neu-overexpressing breast cancer cells [7], it is not known if AS-IRE acts like a canonical IRE in terms of its interaction with IRP and its regulation by iron. Furthermore, it is not yet demonstrated if AS-IRE could direct the expression of a therapeutic gene to achieve a preferential cell killing in HER-2/neu-overexpressing cancer cells. In this report, we showed that AS-IREs interact with endogenous IRP and repress translation in vitro. Moreover, we found that AS-IRE-directed gene expression is regulated by iron in low HER-2/neu-expressing breast cancer cells but not in high HER-2/neu-expressing breast cancer cells. Importantly, we showed that by using AS-IRE to direct the expression of a pro-apoptotic protein, Bax [11], we could achieve preferential cell killing in breast cancer cells that overexpress HER-2/neu mRNA. Thus, our results suggest a potential application of the AS-IRE-mediated gene expression system to targeting HER-2/neu-overexpressing breast cancer cells.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines, MDA-MB-453 and MDA-MB-468, were cultured in complete medium, i.e. DMEM/F12 and 10% fetal bovine serum.

2.2. Plasmid construction

To make a luciferase expression plasmid (luc), a luciferase gene obtained from pGEM-Luc (Promega) by HindIII/XhoI digest was subcloned into pcDNA3 (Invitrogen) under the control of bacterial phage T7 promoter. The AS-IRE oligonucleotide sequences (upper strand) used in this study are: AS-IRE-1, 5′-AGCTTCAATCCGCAGCCTCTG**CAGTG**CCAGAGCTGCGGATTGGC-3′; AS-IRE-2, 5′-AGCTTCAGCCACCGGCACAGA**AGCTG**CTCTGTCCGGTGGCTGGC-3′; AS-IRE-3, 5′-AGCTTTGGGCCCTGACCCCAG**CAGTG**CCTGGGTCAGGGCCCAGC-3′; AS-IRE-4, 5′-AGCTTGTCATCAGCT**C**CCACA**CAGTG**CTGTGGAGCTGATGACGC-3′; and AS-IRE-5, 5′-AGCTTTGTCATCGTC**C**TCCAG**CAGTG**ACTGGAGACGATGACAGC-3′. The bold letters are the IRE consensus nucleotides and the underlined regions indicate the antisense sequences of HER-2/neu cDNA. The AS-IRE oligonucleotides are flanked by a HindIII sticky end at 5′ and a NotI sticky end at 3′. The NotI-linearized plasmids were used as templates to generate AS-IRE RNAs using T7 RNA polymerase. In addition, AS-IRE-luc plasmids

could be generated by subcloning the AS-IRE oligonucleotides into the 5′UTR of luc at HindIII and NotI sites. DNA sequences (upper strand) of the positive and the negative controls, i.e. cIRE and mcIRE, respectively, are shown as follows: cIRE: 5′-AGCTTCAATAATTATCGGAAG**CAGTG**CCTTCCATAATTATTGGC-3′ and mcIRE: 5′-AGCTTCAATAATTATGGAAG**CAGT**CCTTCCATAATTATTGGC-3′. cIRE was derived from that located in the 3′UTR of transferrin receptor mRNA [8]. cIRE-luc was generated in the same fashion as that of AS-IRE-luc. The internal control used in the dual luciferase assay was a Renilla luciferase (RL) gene driven by Thymidine Kinase gene promoter (i.e. pRL-TK, Promega). hTERT promoter containing 270 bp upstream sequence from the initiating ATG of the hTERT gene was used to construct hTERT-luc and hTERT-AS-IRE4-luc. Both hTERT-Bax and hTERT-AS-IRE4-Bax were generated in the same fashion.

2.3. Recombinant IRP-1 purification

To obtain IRP-1 protein, pT7-His-hIRP (human IRP-1 cDNA expression vector [12]) was used to transform *E. coli* BL21. We isolated soluble IRP-1 protein from a dense overnight culture grown in LB medium at 25°C. Cells were lysed by freeze/thaw cycles in lysis buffer containing 250 mM NaCl, 0.5% Nonidet P-40, 20 mM Tris–HCl, pH 8.0, followed by sonication. Cell debris was removed from *E. coli* extracts by centrifugation at 4000 rpm for 10 min at 4°C. The supernatant (10 ml) was mixed with 2 ml of Ni-NTA agarose (Qiagen) which had been equilibrated with buffer A (250 mM NaCl, 20 mM Tris, pH 8.0, 400 mM KCl, 0.5% NP-40, 0.5 mM PMSF) for 30 min and then washed once with 20 ml of buffer N (24 mM HEPES, pH 7.6; 150 mM KOAc, 1.5 mM $MgCl_2$, 5% glycerol) with 400 mM KCl, once with buffer N alone, and followed by two washes of buffer N with 5 mM imidazole (Sigma). IRP-1 protein was eluted into buffer N with 50 mM imidazole and stored at −80°C.

2.4. RNA retardation assay

The ^{32}P-labeled cIRE probe for gel retardation assays were generated by in vitro transcription using T7 RNA polymerase (Promega). The DNA templates were linearized by NotI. The gel retardation assay was performed according to the protocol described previously [13]. Briefly, 5 μg of cell lysate was incubated with 100 ng of cold in vitro transcribed cIRE, mcIRE, or AS-IREs at room temperature for 15 min before adding 2 ng of ^{32}P-labeled cIRE probe to the binding reaction for another 15 min. Then, 1 μl RNase T1 (2.5 units/μl) was added to the reaction and incubated for 10 min at room temperature followed by addition of 2 μl of heparin (40 μg/μl) to the reaction and incubated for 10 min at room temperature. The RNA/protein complex was analyzed by non-denaturing 6% PAGE and visualized by autoradiography.

2.5. In vitro translation

In vitro transcription coupled with in vitro translation in wheat germ extract was performed according to T7 TNT® coupled system protocol provided by the manufacturer (Promega). Briefly, 25 μl of wheat germ extract was mixed with 2 μl reaction buffer, 0.5 μl amino acid mixture (without leucine), 0.5 μl amino acid mixture (without methionine), 1 μl RNasin (40 units/μl), 1 μl T7 RNA polymerase, 2 μl linearized plasmid DNA (0.5 μg/μl), with or without purified IRP-1 protein (1.6 μg), and adding H_2O to a total reaction volume of 50 μl. The reactions were incubated at 30°C for 90 min. 2.5 μl of reaction was subsequently used for luciferase activity measurement.

2.6. DNA transfection

The cells were seeded at 1×10^4 cells per well in six-well plates. Liposome-mediated transfection was performed as follows: 2 μg of tested DNA and 0.2 μg of pRL-TK (an internal control) were mixed in 100 μl of serum-free OPTI-MEM medium (Gibco-BRL). DNA was then added drop-wise into 100 μl OPTI-MEM medium containing 0.2 mM liposome. The DNA/liposome complex was incubated at room temperature for 20 min. before added to the cells (which were previously washed with 2 ml of 1× PBS (pH 7.4) and in 800 μl serum-free medium). After 6 h incubation, medium were removed and 2 ml complete medium was added. Each transfection was performed in triplicate. Forty-eight hours after transfection, both luc and RL activities were determined by the Dual Luciferase Reporter Assay System (Promega) using a luminometer (TD-20/20, Promega). The TUNEL assay was performed as described previously [14].

3. Results

To identify potential AS-IREs that could direct preferential gene expression in HER-2/neu-overexpressing cancer cells, we scanned the antisense sequence of HER-2/neu cDNA and searched for regions that contain an IRE consensus sequence, 5′-CNNNNNCAGUG-3′. Five elements on the HER-2/neu antisense sequence fulfilled the IRE consensus requirements, i.e. 458–479 (AS-IRE-1), 825–847 (AS-IRE-2), 1729–1750 (AS-IRE-3), 2904–2924 (AS-IRE-4), and 3193–3215 (AS-IRE-5). (The nucleotide numbering is based on the HER-2/neu cDNA sequence [15].) To test whether AS-IREs could bind to IRP, we performed a competition assay in which the cold AS-IREs were used to compete with the RNA/protein complex formed by the radioactive (^{32}P) labeled control IRE (cIRE) (derived from the IREs located in 3′UTR of transferrin receptor mRNA [8]) and the endogenous IRP from cell lysate of MDA-MB-453 breast cancer cells [16,17] (Fig. 2A, indicated by an arrow). This RNA/protein complex is indeed IRE/IRP complex since it can be competed away by excess (50-fold) amount of cold cIRE but not by mutant IRE (mcIRE) in which both bulge C in the stem and G at the fifth position in the loop have been deleted. When excess amount of cold in vitro synthesized AS-IREs were added to the cIRE/IRP binding reactions, the cIRE/IRP complex was readily competed. This result suggests that these AS-IREs have maintained the integrity of both the sequence and secondary structure that are necessary for IRE/IRP interaction in vitro [18].

To demonstrate functionally an AS-IRE/IRP interaction-mediated translational inhibition, we placed the AS-IREs in the 5′UTR of a luciferase (luc) reporter gene to generate AS-IRE1 ~5-luc driven by the bacterial phage T7 promoter. We tested the in vitro translational inhibitory function of AS-IREs by adding recombinant IRP-1 to a transcription and translation coupling system (TNT) in wheat germ extract (WGE) that does not naturally contain IRP [19,20]. Like the positive control, cIRE-luc (Fig. 2B), a functional AS-IRE allows translation of luc mRNA in WGE without IRP-1, but not with IRP-1. Although AS-IREs direct different level of basal luc activities, our result clearly shows that most AS-IRE-directed translation can be inhibited by IRP-1 with exception of AS-IRE2 and AS-IRE3 (Fig. 2B). As expected, the addition of IRP-1 has little effect on luc. Interestingly, AS-IRE4 appears to be most sensitive to IRP-mediated translational inhibition with more than 18-fold reduction in translational efficiency

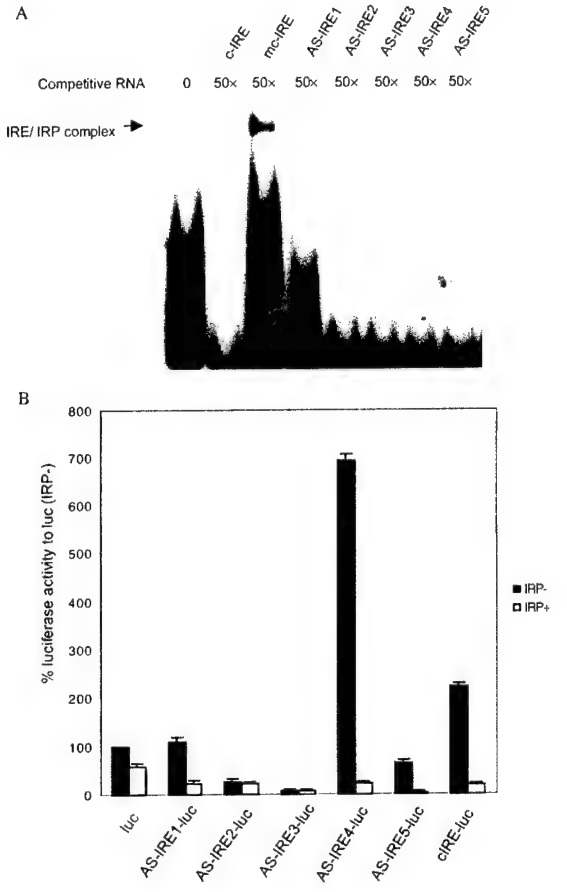

Fig. 2. (A) AS-IREs interact with cellular IRP. The radioactive-labeled cIRE probe (2 ng) incubated with the MDA-MB-453 cell lysate (5 μg) serves as the positive control (0). The cIRE/IRP complex resolved on a 6% native PAGE is indicated by an arrow. The competition assay was performed by incubating 50-fold (100 ng) of cold cIRE, mcIRE, or AS-IRE1 ~5 with the positive control. (B) Recombinant IRP-1 inhibits AS-IRE-mediated translation in vitro. One microgram of linearized luciferase reporter gene (luc, AS-IRE1~5-luc, or cIRE-luc) driven by T7 promoter was used in the in vitro transcription and translation (TNT) system (Promega) in wheat germ extract with or without purified recombinant IRP-1 protein (1.6 μg). The luciferase activity was measured by a luminometer. The relative luciferase activity is shown here and it was determined by setting the activity of luc without IRP-1 at 100%.

(Fig. 2B). Therefore, we chose AS-IRE4-Luc to further characterize the system.

Another prerequisite for a functional IRE is its ability to be regulated by iron. For example, IRE/IRP-directed translation is up-regulated in the presence of iron source, e.g. hemin, but is down-regulated when iron is scarce, e.g. in the presence of an iron chelator, desferrioxamine (Desf) [21]. To test if AS-IRE4 is iron-regulated, we placed AS-IRE4-luc under the control of human telomerase reverse transcriptase (hTERT) gene promoter [22] that has been used to direct tumor-specific gene expression in vitro and in vivo [23]. Thus, hTERT-AS-IRE4-luc was subsequently generated; hTERT-luc was made to serve as a control. We then transfected both constructs into a low HER-2/neu-expressing human breast cancer cell line, MDA-MB-468, with (hemin) or without (Desf) iron, followed by a luciferase assay. As shown in Fig. 3, in the presence of iron, luciferase activity is about 1.5-fold higher than that in the absence of iron. As expected, hTERT-luc is not regulated by iron. Together, our results suggest that AS-IRE4 functions as a canonical IRE in that it interacts with IRP and is regulated by iron.

To test if AS-IRE4 could preferentially direct gene

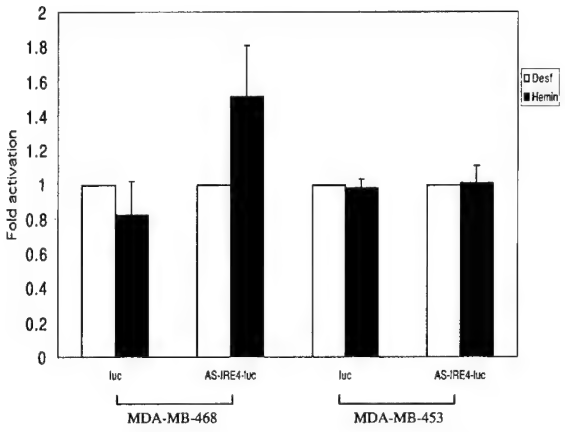

Fig. 3. AS-IRE4-mediated translation is regulated by iron. hTERT-AS-IRE4-luc (AS-IRE4-luc) or hTERT-luc (luc) was transfected into either MDA-MB-453 or MDA-MB-468 cells in the presence of 50 μM hemin (close bars) or 50 μM Desf (open bars). The luciferase activity was measured 48 h after transfection. The internal control, pRL-TK, was used to normalize transfection efficiency. The relative luciferase activity of hTERT-IRE4-luc or hTERT-luc in the presence of hemin was measured against that in the presence of Desf (100%) in each cell line.

expression in a HER-2/neu overexpression-specific manner in vivo, we transfected hTERT-AS-IRE4-luc into either low (e.g. MDA-MB-468) or high (e.g. MDA-MB-453 which overexpresses 12~16-fold HER-2/neu mRNA [24]) HER-2/neu-expressing human breast cancer cell line. Since our model predicts a disruption of AS-IRE stem-loop structure in high HER-2/neu-expressing cells [7], we reasoned that the iron regulation would be subsequently impaired in AS-IRE-directed translation. To test the hypothesis, we transfected hTERT-AS-IRE4-luc (or hTERT-luc) into MDA-MB-453 cells with or without excess iron. As shown in Fig. 3, in contrast to that in MDA-MB-468 cells, no significant difference in AS-IRE4-directed luciferase activity with or without iron was observed in MDA-MB-453 cells. Again, hTERT-luc is not regulated by iron. Thus, this result supports the idea that AS-IRE/IRP interaction is disrupted in cells that overexpress HER-2/neu mRNA. Furthermore, our model also predicts a preferential gene expression on the translational level directed by AS-IRE in HER-2/neu-overexpressing cells [7]. To test that possibility, we transfected hTERT-AS-IRE4-luc (or hTERT-luc) into MDA-MB-468 and MDA-MB-453 cells. Consistent to the translational inhibitory function of IRE, AS-IRE4 drastically reduces gene expression in either cell line, i.e. $<1\%$ in MDA-MB-468 and $<5\%$ in MDA-MB-453 (Fig. 4A). However, the relative luciferase activity of hTERT-AS-IRE4-luc in MDA-MB-453 is six-fold higher than that in MDA-MB-468. The above observation suggests that AS-IRE4 is able to direct a preferential gene expression in HER-2/neu-overexpressing breast cancer cells.

Recently, Bax (a pro-apoptotic protein in the Bcl-2 protein family [11]) has been used as a potential therapeutic gene to achieve anti-tumor effect on both in vitro and in vivo cancer model systems [23]. We then tested if AS-IRE4 could direct Bax gene expression in such a way that would result in a preferential killing of HER-2/neu-overexpressing breast cancer cells. To accomplish this, we placed Bax gene under the control of hTERT promoter with or without AS-IRE4 in 5′UTR to generate hTERT-AS-IRE4-Bax and hTERT-Bax, respectively. We transfected these constructs into either MDA-MB-468 or MDA-MB-453 cells, followed by TUNEL assay [14] to monitor apoptosis. Consistent with the result from the reporter assay (Fig. 4A), we observed a significantly low Bax-

induced cell killing by hTERT-AS-IRE4-Bax as compared with that by hTERT-Bax, i.e. ~10% in MDA-MB-468 and ~24% in MDA-MB-453 cells (Fig. 4B). Again, it supports the translational inhibitory function of AS-IRE4. However, there is two-fold more apoptosis caused by hTERT-AS-IRE4-Bax transfection in MDA-MB-453 than that in MDA-MB-468 cells. This observation supports the hypothesis that hTERT-AS-IRE4-Bax could be used to achieve a preferential cell killing in breast cancer cells that overexpress HER-2/neu mRNA.

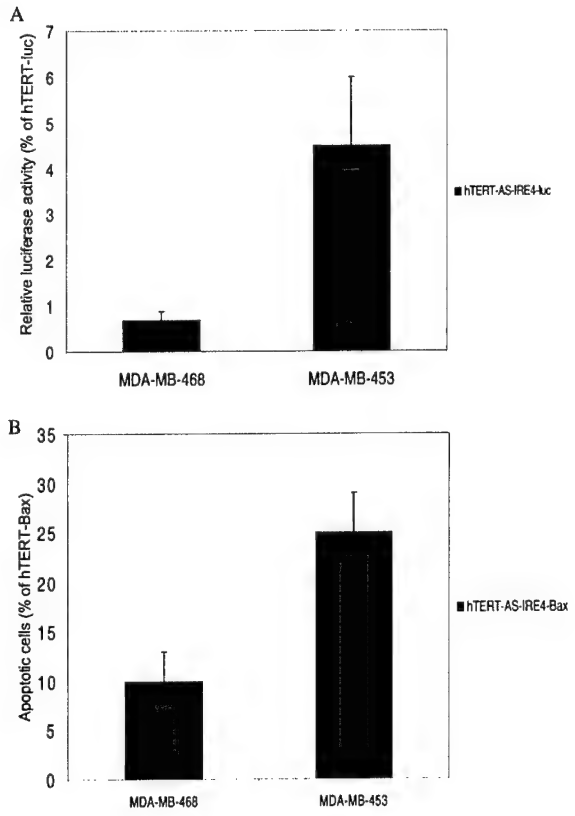

Fig. 4. (A) AS-IRE4 mediates preferential gene expression in HER-2/neu-overexpressing breast cancer cells. hTERT-luc and hTERT-IRE4-luc were transfected into either MDA-MB-453 or MDA-MB-468 cells. The luciferase activity was measured 48 h after transfection and the internal control, i.e. pRL-TK, was used to normalize the transfection efficiency. The relative lucifease activity of hTERT-IRE4-luc was measured against the activity of hTERT-luc (100%) in each cell line. (B) AS-IRE4 mediates preferential cell killing in HER-2/neu-overexpressing breast cancer cells. hTERT-Bax and hTERT-IRE4-Bax were transfected into either MDA-MB-453 or MDA-MB-468 cells (1×10^6 cells per 60 mm dish). Forty-eight hours after transfection, cells were harvested into a cytospin chamber. TUNEL assay was then performed to score the apoptotic cells under 40× magnification from 10 random fields with total more than 300 cells. Each sample was counted twice. The percentage of apoptotic cell per field was calculated and the average of ten fields is presented. The relative cell killing of hTERT-IRE4-Bax is measured against that of hTERT-Bax (100%) in each cell line.

4. Discussion

In this report, we demonstrated that, like the canonical IRE, AS-IREs bind to IRP. In addition, when placed in the 5′UTR of a reporter gene, they (with exception of AS-IRE2 and AS-IRE3) behave as translational inhibitors in vitro. Using hTERT-AS-IRE4-luc, we demonstrated that AS-IRE4 is regulated by iron in low HER-2/neu-expressing MDA-MB-468 cells but such regulation is impaired in high HER-2/neu-expressing MDA-MB-453 cells. Thus, this observation is consistent to our working hypothesis [7] that AS-IRE4/IRP interaction would be abolished HER-2/neu-overexpressing cells. Therefore, our results strongly suggest that AS-IRE4 is a functional IRE. Importantly, we showed that AS-IRE4 could preferentially direct gene expression in HER-2/neu-overexpressing breast cancer cells. Specifically, we demonstrated a preferential reporter gene expression of hTERT-AS-IRE4-luc and a preferential killing with hTERT-AS-IRE4-Bax of HER-2/neu-overexpressing MDA-MB-453 cells as opposed to MDA-MB-468 cells.

Obviously, further experiments are required to improve the targeting efficiency in terms of increasing the AS-IRE-directed differential expression in HER-2/neu-overexpressing cancer cells. It is worth noting that we have attempted to enhance targeting specificity in HER-2/neu-overexpressing cancer cells by lengthening the lower stems of AS-IREs. The resulting lengths of sense/antisense hybrids are 29-, 36-, and 70-bp as opposed to 21-bp in the unmodified AS-IREs used in this study. The modified AS-IREs showed a reduced translational efficiency as determined by luciferase reporter assay in a length-dependent, but HER-2/neu level-independent manner (data not shown). We found that the longer the sense/antisense hybrid leads to the lower translation efficiency regardless of the HER-2/neu level in the cell. It is likely that the long sense/antisense hybrids them-

selves may become translational blocks and/or the targets of certain double-stranded RNA-specific ribonucleases. Thus, this result suggests the existence of a critical length of sense/antisense hybrid between AS-IRE and HER-2/neu mRNA required for an optimal preferential translation in HER-2/neu-overexpressing cancer cells. Another way to improve the targeting efficiency is to screen more potential AS-IREs on the HER-2/neu antisense sequence. For example, instead of limiting to the consensus 5′-**C***NNNNN***CAGUG**-3′, one could find shorter sequence like 5′-**C***NNNNN***CAG**-3′ in HER-2/neu antisense sequence, e.g. 5′-**CTG***GACAT***G**-3′ (+277~ + 285) of HER-2/neu sense cDNA sequence, this sense/antisense hybrid, if formed, would also prevent the formation of IRE. Alternatively, one may design AS-IRE in a way that would hybridize HER-2/neu mRNA at different region of AS-IRE, e.g. the 3′ half as opposed to the 5′ half used in this study, since it is known that the target site on a RNA secondary structure is also critical to achieve a better targeting efficiency by antisense oligonucleotides [25].

Once an optimal AS-IRE-based gene expression system is established, using hTERT (or other tumor-specific) promoter to drive a therapeutic gene such as Bax, it is likely to achieve a tumor-specific killing in cancer cells that overexpress HER-2/neu mRNA. The AS-IRE approach thus represents a novel gene expression strategy that may preferentially target cancer cells that overexpress a known gene.

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Proapoptotic and Antitumor Activities of Adenovirus-mediated p202 Gene Transfer[1]

Yi Ding, Yong Wen, Bill Spohn, Li Wang, Weiya Xia, Ka Yin Kwong, Ruping Shao, Zheng Li, Gabriel N. Hortobagyi, Mien-Chie Hung, and Duen-Hwa Yan[2]

Departments of Molecular and Cellular Oncology [Y. D., Y. W., B. S., L. W., W. X., K. Y. K., R. S., Z. L., M-C. H., D-H. Y.], Breast Medical Oncology [G. N. H.], and Surgical Oncology [M-C. H., D-H. Y.]. The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Purpose and Experimental Design: **p202, a mouse IFN-inducible protein, is a member of the 200-amino acid repeat family. Enforced p202 expression in stable cancer cell lines resulted in growth inhibition *in vitro* and tumor suppression *in vivo*. However, to study the immediate effect of p202 and test the potential efficacy of p202 treatment, an efficient gene delivery system for p202 is required. For these purposes, an adenoviral vector expressing the p202 gene (Ad-p202) was generated. We examined the effects of Ad-p202 infection on human breast cancer cells. Furthermore, we tested the efficacy of Ad-p202 treatment on breast and pancreatic cancer xenograft models.**

Results: **We found that Ad-p202 infection induces growth inhibition and sensitizes the otherwise resistant cells to tumor necrosis factor α-induced apoptosis. In addition, we demonstrated for the first time that Ad-p202 infection induces apoptosis and that activation of caspases is required for the full apoptotic effect. More importantly, we showed the efficacy of Ad-p202 treatment on breast cancer xenograft models, and this antitumor effect correlated well with enhanced apoptosis in Ad-p202-treated tumors.**

Conclusions: **We conclude that Ad-p202 is a potent growth-inhibitory, proapoptotic, and tumor-suppressing agent. Ad-p202 may be further developed into an efficient therapeutic agent for human cancer gene therapy.**

INTRODUCTION

IFN is known to exert antiproliferative and antiviral actions. It has both direct and indirect (immunological) antitumor activity in several human malignancies, including leukemia and lymphomas as well as solid tumors. Aside from the therapeutic effects of IFN in certain clinical settings, there are also undesirable side effects (*e.g.*, fever, chills, anorexia, and anemia) associated with the high-dose IFN treatment that is often required to obtain a significant response (1, 2). This has hampered IFN as an effective anticancer agent. In an attempt to circumvent this potential drawback and maintain the benefit of IFN-mediated antitumor activity, we have begun to explore the possibility of using an IFN-inducible protein, p202, as a potential therapeutic agent (3–5). p202 is a mouse IFN-inducible, chromatin-associated protein. It belongs to the 200-amino acid repeat family (6, 7). The unique feature of p202 is illustrated by its ability to interact with several important transcriptional regulators that include E2Fs, Rb, pocket proteins p130 and p107, Fos/Jun, c-Myc, NF-κB,[3] and p53BP-1 (reviewed in Ref. 8), resulting in transcriptional repression of genes that are upregulated by these transcriptional regulators. The exact role of p202 in the IFN-mediated signal pathway is not well defined. However, consistent with the multiple antitumor activities of IFN (9), enforced expression of p202 in stable murine fibroblasts and human cancer cell lines leads to retardation of cell growth and suppression of transformation phenotype (3, 5, 10, 11). Furthermore, breast cancer cells stably transfected with p202 are sensitized to TNF-α-induced apoptosis (5), and that effect is associated with inactivation of the TNF-α-induced NF-κB via p202-NF-κB interaction. We postulated that p202 sensitizes cancer cells to TNF-α-induced apoptosis by inactivating NF-κB, which, in turn, turns off NF-κB-activated antiapoptotic gene expression, leading to enhanced TNF-α-induced cell killing (5).

To generate a p202-based therapeutic agent for efficacy study in animal models and a tool to study the biological function of p202, we constructed Ad-p202. In this study, we

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[2] To whom requests for reprints should be addressed, at Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3677; Fax: (713) 794-0209; E-mail: dyan@mdanderson.org.

[3] The abbreviations used are: NF-κB, nuclear factor κB; TNF-α, tumor necrosis factor α; i.t., intratumor; CMV, cytomegalovirus; GFP, green fluorescence protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MOI, multiplicity of infection; PARP, poly-(ADP-ribose) polymerase; pfu, plaque-forming unit(s); TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PI, postinfection; fmk, fluoromethyl ketone; Z-VAD, N-benzyloxycarbonyl-Val-Ala-Asp; Z-DEVD, N-benzyloxycarbonyl-Asp-Glu-Val-Asp.

show that Ad-p202 infection of breast cancer cells resulted in growth inhibition and sensitization to TNF-α-induced apoptosis. Interestingly, we found that Ad-p202 infection alone induces apoptosis in breast cancer cells, and the activation of caspases is critical for this process. More importantly, we demonstrated the efficacy of Ad-p202 treatment in human breast cancer xenograft models through either i.t. or i.v. injection. This antitumor activity correlated well with p202 expression and apoptosis in Ad-p202-treated tumors. Together, our results suggest that Ad-p202 is a potent growth-inhibitory, proapoptotic, antitumor agent that could be further developed to become an effective therapeutic agent for cancer gene therapy treatment.

MATERIALS AND METHODS

Generation of Ad-p202. Ad-p202 was constructed according to the protocol described previously (12). p202 cDNA (11) was subcloned into an adenovirus vector (pAdTrack-CMV) that carries a CMV promoter-driven GFP. A separate CMV promoter directs p202 cDNA. A control virus, an adenoviral vector expressing luciferase gene and GFP (Ad-Luc), was likewise generated. The expression of GFP gene enabled us to monitor the infection efficiency by direct observation using a fluorescence microscope.

***In Vitro* Growth Assays.** MDA-MB-468 human breast cancer cells were maintained in DMEM/Ham's F-12 (HyClone Laboratories, Inc.) supplemented with 10% (v/v) fetal bovine serum. MTT is a pale yellow substrate that can be cleaved by living cells (but not dead cells) to yield a dark blue formazan product. The extent of MTT cleavage determined colorimetrically (at 570 nm) can be used to measure cell proliferation. Briefly, 2×10^3 cells were plated in 96-well culture plates in 0.1 ml of culture medium. Ad-p202 or Ad-Luc was added at a MOI of 200 on the next day. At the different times indicated, 20 μl of MTT (5 mg/ml stock solution) were added to each well. Cells were cultured for an additional 2 h, and then 100 μl of lysis buffer [20% SDS in 50% *N,N*-dimethylformamide (pH 4.7)] were added to each well, followed by 5 h of incubation, and then absorbance was measured at 570 nm. [^{3}H]Thymidine incorporation assay was performed as described previously (13).

Apoptosis Assays. For flow cytometry analysis, cells were collected at the indicated times PI, washed once with PBS, and suspended in 0.5 ml of PBS containing 0.1% (v/v) Triton X-100 for nuclei preparation. The suspension was filtered through a nylon mesh and then adjusted to a final concentration of 0.1% (w/v) RNase and 50 μg/ml propidium iodide. Apoptotic cells were quantified by FACScan cytometer. The DNA fragmentation assay was carried out as described previously (13).

Western Blot Analysis. MDA-MB-468 cells treated with or without TNF-α (R&D Systems, Inc., Minneapolis, MN) were infected with Ad-p202 or Ad-Luc at a MOI of 200. Seventy-two h PI, cells were lysed with radioimmunoprecipitation assay lysis buffer. The protein extracts were subjected to SDS-PAGE followed by Western blotting according to the procedure described previously (5). Goat anti-p202 polyclonal antibody and anti-PARP antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Transduction Laboratories (Lexington, KY), respectively. Caspase inhibitors Z-VAD and Z-DEVD-fmk were purchased from Enzyme Systems Products (Livermore, CA).

Gel-Shift Assay. The NF-κB gel-shift assay was performed as described previously (13).

Ad-p202 Gene Therapy in Human Cancer Xenograft Models. For the orthotopic breast cancer xenograft model, MDA-MB-468 cells (2×10^6 cells) were implanted in mammary fat pads (2 tumors/mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups: group 1, Ad-Luc; and group 2, Ad-p202. For i.t. injection, 1×10^9 pfu viruses/treatment was administered. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for 7 weeks and once a week thereafter. For tail vein injection, 5×10^8 pfu viruses/treatment were administered. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for 5 weeks and once a week thereafter.

Immunohistochemical Analysis of p202 Expression and Apoptosis. Mice were sacrificed 24 h after the last treatment. Tumors obtained from Ad-p202- or Ad-Luc-treated mice bearing either breast or pancreatic tumors were then excised and fixed with formalin and embedded in paraffin. Immunohistochemical analysis of p202 protein expression was performed according to the protocol described previously (14). Tumor sections were incubated with goat polyclonal antibody specific for p202 (Santa Cruz Biotechnology) followed by incubation with biotinylated rabbit antigoat IgG and subsequent incubation with avidin-biotin peroxidase before visualization. TUNEL assay was performed to detect the ends of degraded DNA fragments induced by apoptosis according to the protocol described previously (15).

RESULTS

Ad-p202 Mediates p202 Expression in Breast Cancer Cells. To test the efficiency and monitor the expression of p202 protein by Ad-p202 infection, we infected MDA-MB-468 breast cancer cells with either Ad-p202 or Ad-Luc followed by fluorescence microscopy and Western blot analysis, respectively. As shown in Fig. 1*A*, Ad-p202 and Ad-Luc infection at a MOI of 200, at 24-h PI, exhibited >90% infection efficiency as indicated by the GFP-positive cells shown in a representative field (Fig. 1*A*, *right panels*). The same cells are shown in phase-contrast images (Fig. 1*A*, *left panels*). The mock-infected cells (*Control*) showed no GFP expression. In addition to MDA-MB-468, we found that Ad-p202 could infect a panel of other human breast cancer cell lines (*e.g.*, MDA-MB-453, MDA-MB-435, MDA-MB-231, and MCF-7), albeit with various infection efficiency rates (data not shown). We chose the MDA-MB-468 cell line for subsequent studies because it is tumorigenic in the mouse xenograft model and has relatively high infection efficiency by Ad-p202. The expression of p202 protein in Ad-p202-infected cells was further analyzed by Western blot using p202-specific antibody. Fig. 1*B* shows that whereas the mock- and Ad-Luc-infected cells have no p202 expression, Ad-p202 infection efficiently directed p202 expression in MDA-MB-468 cells in a dose-dependent manner. These results clearly demonstrate that Ad-p202 infection adequately directs p202 expression in MDA-MB-468 cells.

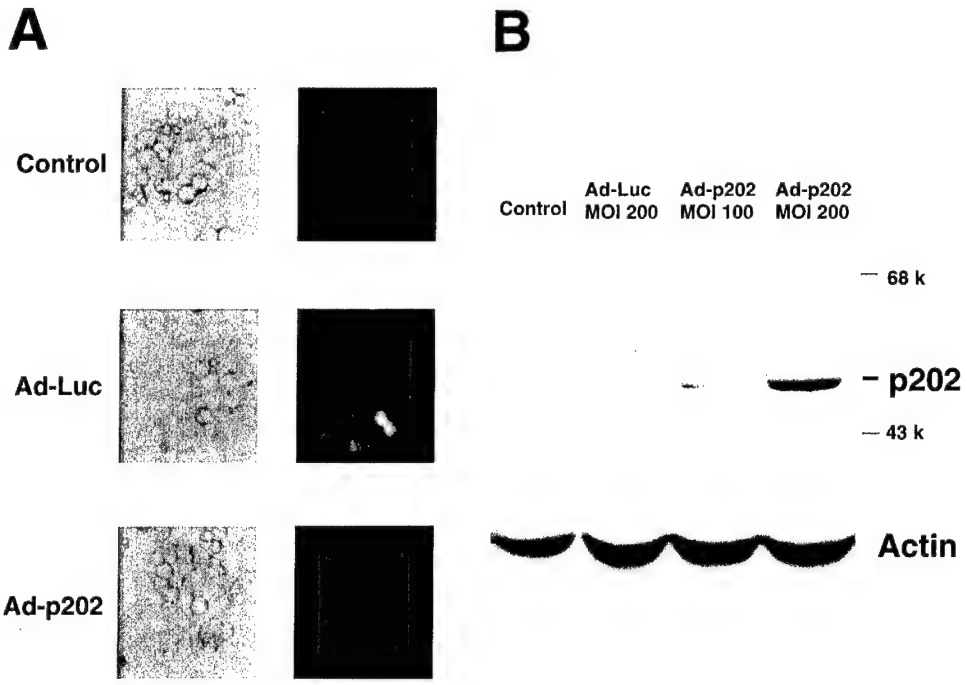

Fig. 1 Ad-p202 construction, p202 expression, and infection efficiency. *A*, Ad-p202 was generated according to the protocol described previously (12). The pAdTrack-CMV vector contains two independent CMV promoter-driven transcription units, one for GFP and one for p202 cDNA. MDA-MB-468 human breast cancer cells were infected by Ad-Luc or Ad-p202 at a MOI of 200. Twenty-four h PI, >90% of cells were found to be GFP positive as visualized by fluorescence microscopy (*right panels*), indicating that the infection efficiency is >90%. *Left panels*, phase-contrast microscopy. *Control*, mock-infected cells. *B*, p202 protein is expressed in Ad-p202 infected cells. MDA-MB-468 cells infected with Ad-Luc or Ad-p202 for 72 h were analyzed for p202 protein expression by Western blot. *Control*, mock-infected cells. Actin protein was used as an equal loading control.

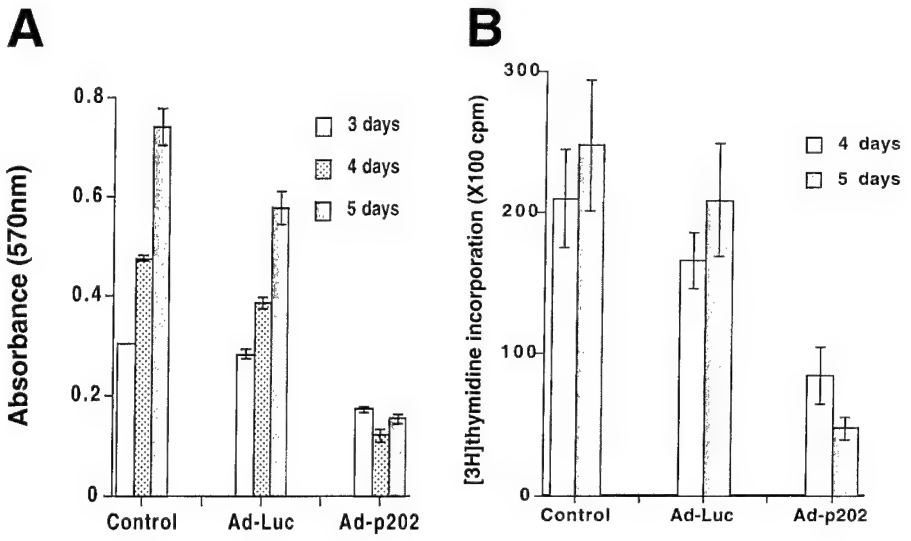

Fig. 2 Ad-p202 infection inhibits cell proliferation. MDA-MB-468 cells were infected with Ad-Luc or Ad-p202 at a MOI of 200. Cell growth was monitored at the indicated PI time (3–5 days) by (*A*) MTT assay [means ± SD (n = 3)] or (*B*) [^{3}H]thymidine incorporation assay; data are presented as the means of quadruplicates.

Ad-p202 Infection Reduces Breast Cancer Cell Growth. To assess the effect of Ad-p202 infection on cell growth, we infected MDA-MB-468 cells with either Ad-p202 or Ad-Luc followed by *in vitro* growth assays such as the MTT assay and [^{3}H]thymidine incorporation assay at different time points, *i.e.*, 3–5 days PI. As shown in Fig. 2, whereas the mock infection (*Control*) and Ad-Luc infection have no growth-inhibitory effect on MDA-MB-468 cells, Ad-202 infection significantly hampered cell growth (Fig. 2*A*) and DNA synthesis rates (Fig. 2*B*). This observation strongly indicates that Ad-p202 infection inhibits cell growth in breast cancer cells and is congruent with our previous findings using stable cancer cell lines (3, 5).

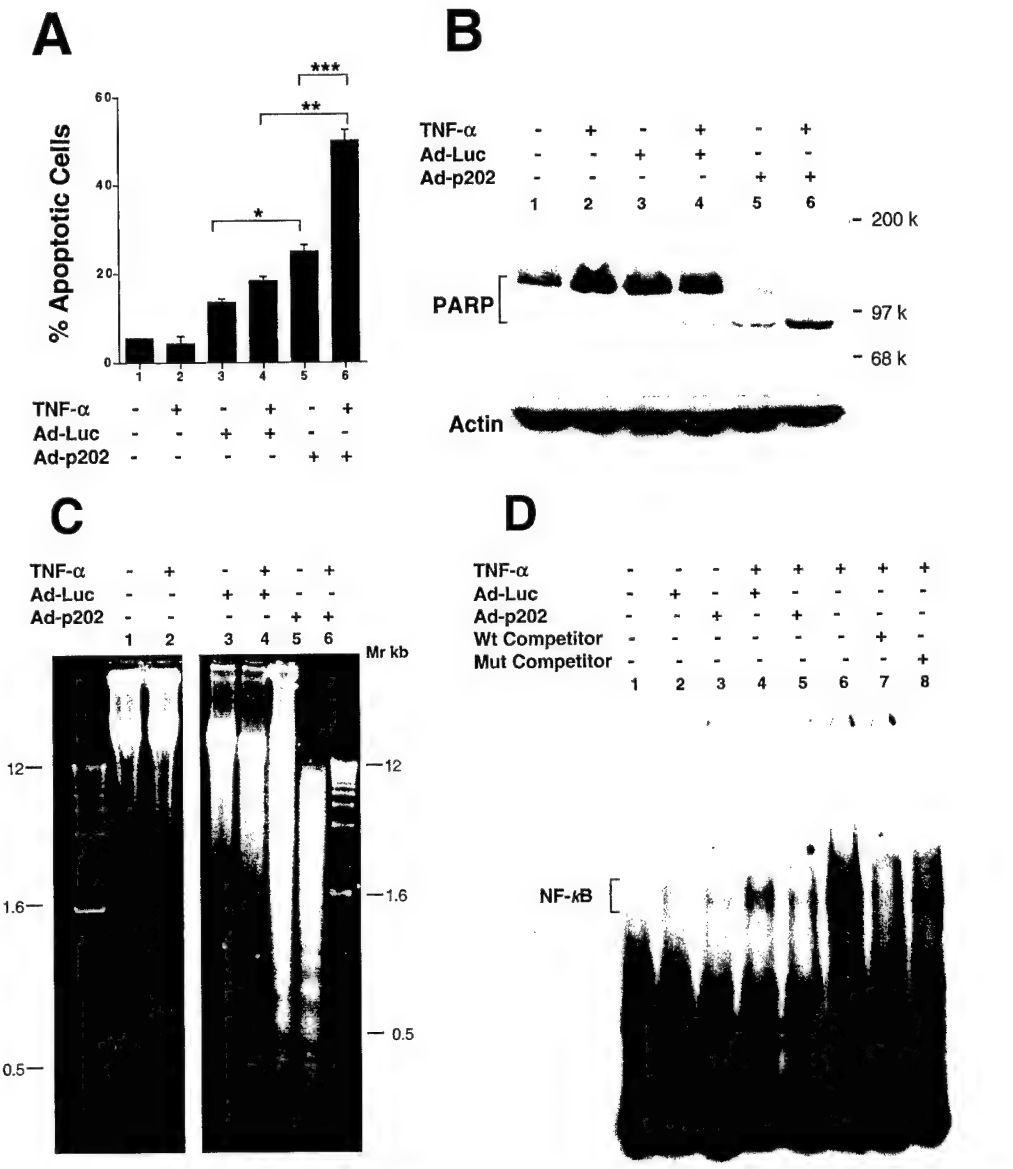

Fig. 3 Ad-p202 infection induces apoptosis and sensitizes cells to apoptosis induced by TNF-α. MDA-MB-468 cells were infected with Ad-Luc or Ad-p202 at a MOI of 200. *A*, 24 h PI, TNF-α (50 ng/ml) was added to the medium and incubated for 48 h (72 h PI). Apoptosis was then monitored by flow cytometry analysis (done in triplets; *bars*, SD). *t* test: *, $P < 0.001$; **, $P < 0.0002$; and ***, $P < 0.0005$. *B*, PARP cleavage assay. Twenty-four h PI, TNF-α (50 ng/ml) was added to the medium and incubated for 24 h. Treated cells were harvested 48 h PI. The PARP protein (M_r 116,000) was cleaved into M_r 85,000 product in the event of apoptosis. *C*, DNA fragmentation assay. Twenty-four h PI, TNF-α (50 ng/ml) was added to the medium and incubated for 24 h. Treated cells were harvested 48 h PI. *D*, Ad-p202 infection inhibits TNF-α-induced NF-κB DNA binding activity. MDA-MB-468 cells were infected with Ad-p202 or Ad-Luc in the presence or absence of TNF-α (50 ng/ml) 24 h PI for 30 min. The nuclear extracts were then isolated and incubated with a radioactive-labeled oligonucleotide containing NF-κB binding site (13). The excess cold wild-type or mutant NF-κB binding site was added to the incubation to demonstrate the specific NF-κB DNA binding activity. The NF-κB-DNA complex is indicated.

Ad-p202 Infection Induces Apoptosis in Breast Cancer Cells. Without stress signals, the p202 stable cancer cell lines do not exhibit apoptotic phenotype (3, 5). It is possible that p202 stable cell lines isolated after a vigorous selection process may possess a physiologically tolerant level of p202. The fact that only a small number of p202 stable cell lines were obtained by colony-forming assay (3, 5) raises the possibility that p202 expression alone may induce apoptosis. To test that possibility, we infected MDA-MB-468 cells with Ad-p202 or Ad-Luc followed by flow cytometry analysis at 72-h PI to detect apoptosis by measuring the cell population in the sub-G_1 phase of cell cycle. As shown in Fig. 3*A*, although Ad-Luc infection induced modest apoptosis (compare *Lane 1* with *Lane 3*), Ad-p202 infection (*Lane 5*) caused significantly more apoptosis (>20%) than Ad-Luc infection (*Lane 3*; $P < 0.001$, *t* test). That observation was further confirmed by two other apoptosis assays: (*a*) the PARP cleavage assay, in which full-length PARP (M_r 116,000) is cleaved by caspases into a fragment of approximately M_r 85,000 (Fig. 3*B*); and (*b*) a DNA fragmentation assay that is based on the activated endonucleases during apoptosis (Fig. 3*C*). Ad-p202 infection resulted in a marked increase of the PARP cleavage product (M_r 85,000; Fig. 3*B*, *Lane 5* and Fig. 4*A*, *Lane 3*) and an enhancement of DNA fragmentation (Fig. 3*C*, *Lane 5*). In contrast, Ad-Luc infection yielded a minimum amount of M_r 85,000 PARP cleavage product (Fig. 3*B*, *Lane 3* and Fig. 4*A*, *Lane 2*) as well as a near basal level of DNA fragmentation (Fig. 3*C*, compare *Lanes 1* and *3*). Together, our results strongly indicate that Ad-p202 alone induces apoptosis in MDA-MB-468 cells. Given that the MDA-MB-468 cell line harbors mutant p53 (16), Ad-p202-mediated apoptosis thus appears to be independent of p53 status.

p202-mediated Apoptosis Is Caspase-dependent. Because caspases are activated during apoptosis and have a variety

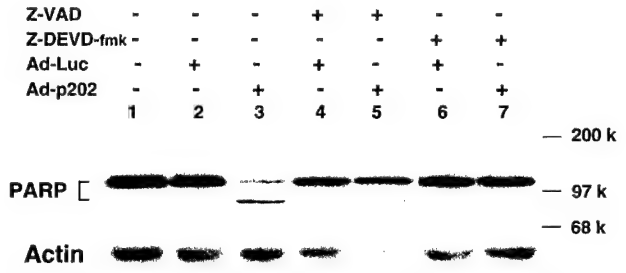

Fig. 4 The activation of caspases is critical for Ad-p202-mediated apoptosis. Z-VAD (100 μM) and Z-DEVD-fmk (80 μM) inhibit Ad-p202-mediated apoptosis in MDA-MB-468 cells. Western blot analysis of PARP cleavage and actin expression was performed 48 h PI. The intensity of full-length PARP and PARP cleavage product bands was measured using NIH Image 1.62 software.

of substrates including PARP (17), the cleavage of PARP in Ad-p202-infected cells suggests that the activation of caspase may be involved in Ad-p202-induced apoptosis. To test that hypothesis, we infected MDA-MB-468 cells with Ad-p202 or Ad-Luc in the presence or absence of a pan-caspase inhibitor, Z-VAD. At 48 h PI, the intensity of full-length PARP and PARP cleavage product bands on Western blot was measured using NIH Image 1.62 software. The percentage of M_r 85,000 product was calculated by setting the total intensity of both M_r 116,000 and M_r 85,000 bands in each lane at 100%. As shown in Fig. 4, the addition of Z-VAD attenuates Ad-p202-induced apoptosis, as indicated by the reduced (but not completely eliminated) level of PARP M_r 85,000 cleavage product from 57.4% (*Lane 3*) to 13.3% (*Lane 5*), whereas Z-VAD has no effect on PARP cleavage in Ad-Luc-infected cells (*Lane 4*). This result supports the idea that the activation of caspases, at least in part, is required for Ad-p202 to induce full apoptotic effect. Because PARP is also a substrate for caspase-3, which is considered to be a crucial enzyme commonly activated during apoptosis (17), we examined whether the activation of caspase-3 plays a role in Ad-p202-induced apoptosis. To that end, we infected MDA-MB-468 cells with either Ad-p202 or Ad-Luc in the presence of a caspase-3-specific inhibitor, Z-DEVD-fmk (18). As shown in Fig. 4, the level of PARP cleavage product in Ad-p202-infected MDA-MB-468 cells is significantly reduced to 8.9% (*Lane 7*) with Z-DEVD-fmk treatment as compared with that without Z-DEVD-fmk (57.4%; *Lane 3*). As a control, no detectable PARP cleavage was observed in Ad-Luc-infected cells treated with Z-DEVD-fmk (*Lane 6*). Thus, this result suggests that the activation of caspase-3 is critical for Ad-p202-mediated apoptosis. To further confirm this observation, we infected a caspase-3-null breast cancer cell line, MCF-7 (19), with Ad-p202 or Ad-Luc, followed by flow cytometry analysis. We observed that although p202 was readily expressed as determined by Western blot, the Ad-p202-infected MCF-7 cells yielded the similar level of apoptosis as that of the controls, *i.e.*, mock and Ad-Luc infection (data not shown). Therefore, our data suggest that the activation of caspases is critical for Ad-p202 to exert full apoptotic effect.

Ad-p202 Infection Sensitizes Breast Cancer Cells to TNF-α-induced Apoptosis. We tested whether Ad-p202 infection could also sensitize breast cancer cells to TNF-α-induced apoptosis (5). Although MDA-MB-468 cells appear to be resistant to TNF-α (50 ng/ml; added at 24 h PI for 48 h)-induced apoptosis (Fig. 3*A*, *Lanes 1* and *2*; Fig. 3*B*, *Lanes 1* and *2*; and Fig. 3*C*, *Lanes 1* and *2*), the combination of TNF-α and Ad-p202 induced massive cell killing [compare Fig. 3*A*, *Lanes 5* and *6* ($P < 0.0005$); Fig. 3*B*, *Lanes 5* and *6*; and Fig. 3*C*, *Lanes 5* and *6*]. These results suggest that Ad-p202 infection sensitizes MDA-MB-468 cells to TNF-α-induced apoptosis. In contrast, the apoptosis resulting from the combined treatment of TNF-α and Ad-Luc (Fig. 3*A*, *Lane 4*) was significantly less than that from combined treatment with TNF-α and Ad-p202 (Fig. 3*A*, *Lane 6*; $P < 0.0002$). This observation was confirmed by PARP cleavage and DNA fragmentation assays (Fig. 3*B*, *Lanes 4* and *6*; Fig. 3*C*, *Lanes 4* and *6*). These data indicate that the sensitization to TNF-α-induced apoptosis is specific to p202 expression. Because p202-mediated sensitization to TNF-α-induced apoptosis correlated with the inactivation of NF-κB, specifically, via the loss of NF-κB DNA binding activity (5), we next tested whether Ad-p202 infection affects TNF-α-induced NF-κB DNA binding activity. At 24 h PI, TNF-α was added for 30 min, and the nuclear extract was then isolated and subsequently incubated with a radioactive-labeled oligonucleotide containing the NF-κB binding sites. A gel-shift assay was then performed to detect the NF-κB DNA binding activity. As shown in Fig. 3*D*, we observed a complete abolishment of TNF-α-induced NF-κB DNA binding activity in Ad-p202-infected MDA-MB-468 cells (Fig. 3*D*, compare *Lanes 1, 3, 5,* and *6*). As controls, TNF-α-induced NF-κB DNA binding activity (*Lane 6*) can be readily competed by cold wild-type NF-κB DNA binding site (*Lane 7*) and, to a lesser extent, by cold mutant probe (*Lane 8*). Ad-Luc infection also reduces the TNF-α-induced NF-κB DNA binding activity somewhat, but to a lesser extent than Ad-p202 (*Lanes 4* and *5*). Together, our data suggest that Ad-p202 infection could sensitize otherwise resistant MDA-MB-468 cells to apoptosis induced by TNF-α, which correlates with a loss of TNF-α-induced NF-κB DNA binding activity.

Antitumor Activity of Ad-p202 in Cancer Xenograft Models. To test the efficacy of Ad-p202 treatment in an orthotopic breast cancer xenograft model, we implanted MDA-MB-468 cells (2×10^6 cells) into mammary fat pads of female nude mice. Treatment began when tumor size reached ~0.5 cm in diameter (about 2 weeks after implantation). We then treated tumor-bearing mice (7 tumors/treatment group) with either Ad-p202 or control virus Ad-Luc (1×10^9 pfu/treatment) via i.t. injection. Treatments were administered twice per week for 7 weeks and once a week thereafter. Tumor size was measured by using the following formula: tumor size = $1/2 \times L \times S^2$, where L and S are the longest and shortest diameters measured, respectively. The tumor size distribution with Ad-p202 or Ad-Luc treatment at two time points (day 25 and day 67) is presented. Whereas there was little difference at the early stage of treatment (Fig. 5*A*, day 25; $P = 0.13$), the Ad-p202-treated tumors grew significantly slower than those treated with Ad-Luc on day 67 ($P = 0.04$). This result supports the idea of a p202-based gene therapy in breast cancer treatment. Because breast cancer is a metastatic disease, it is critical to develop a systemic delivery system for p202 gene transfer. Although the antitumor effect by i.t. treatment is encouraging, no report has shown a therapeutic effect by systemic administration of p202 in a cancer xenograft model. We then performed systemic gene therapy

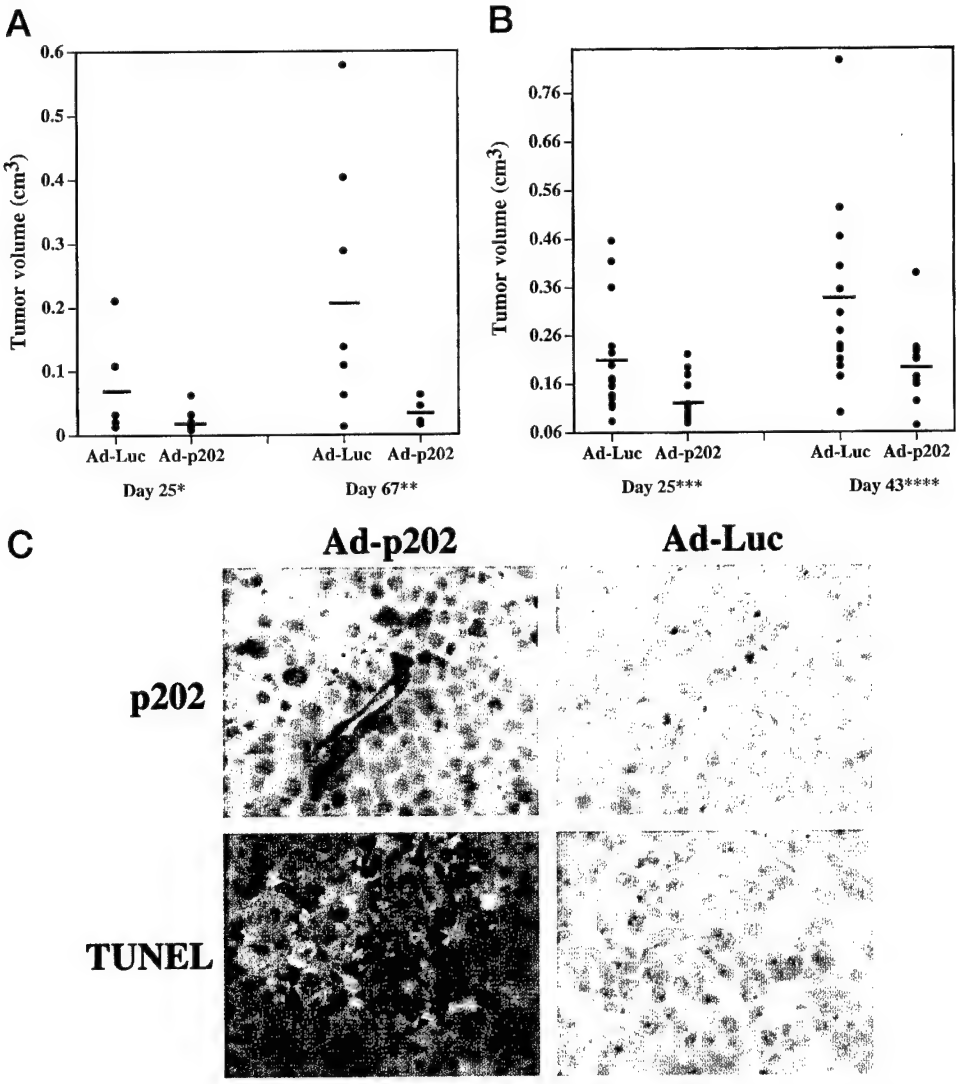

Fig. 5 Antitumor effect by systemic delivery of Ad-p202 in an orthotopic breast cancer xenograft model. *A*, Ad-p202-mediated antitumor effect on breast cancer xenografts by i.t. treatment. MDA-MB-468 cells (2×10^6 cells) were implanted in mammary fat pads of each female nude mouse. Tumor-bearing mice were divided into two treatment groups, Ad-Luc (total, 7 tumors) and Ad-p202 (total, 7 tumors), at 1×10^9 pfu/treatment via i.t. injection. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for 7 weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, *i.e.*, day 25 and day 67. *t* test: *, $P = 0.13$; and **, $P = 0.04$. *B*, Ad-p202-mediated antitumor effect on breast cancer xenografts by systemic treatment. MDA-MB-468 cells (2×10^6 cells) were implanted in mammary fat pads (2 tumors/mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups, Ad-Luc (total, 14 tumors) and Ad-p202 (total, 14 tumors), at 5×10^8 pfu via tail vein injection. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for 5 weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, *i.e.*, day 25 and day 43. *t* test: ***, $P = 0.0097$; and ****, $P = 0.014$. *C*, apoptosis correlates with p202 expression in Ad-p202-treated breast tumors. Mice were sacrificed 24 h after the last systemic treatment as described above. Tumors were then excised and fixed for the subsequent immunohistochemical analysis. p202 expression was analyzed by using an antibody specific for p202 on tumor samples obtained from Ad-p202- or Ad-Luc-treated mice (14). The TUNEL assay was also performed to detect apoptotic cells in these tumors (15). The *arrows* indicate the representatives of apoptotic cells.

experiments by treating tumor-bearing mice with Ad-p202 or Ad-Luc (5×10^8 pfu/treatment, 14 tumors/group) through tail vein injection. Treatments were administered twice per week for 5 weeks and once a week thereafter. As shown in Fig. 5*B*, Ad-p202-treated mice had a significantly reduced tumor growth rate as compared with the Ad-Luc-treated mice on day 25 ($P = 0.0097$) and day 43 ($P = 0.014$). The above-mentioned observation strongly suggests the feasibility of using a systemic p202-based gene therapy treatment for breast cancer. Because Ad-p202 induces apoptosis *in vitro* (Fig. 3), it is likely that the observed antitumor activity may correlate with enhanced apoptosis in Ad-p202-treated tumors. To test this possibility, we examined the presence of apoptosis in breast tumors treated systemically with Ad-p202. Immunostaining for p202 protein and apoptotic cells were performed 24 h after the last Ad-p202 and Ad-Luc treatment. As shown in Fig. 5*C*, p202 expression was readily detected by immu-

nohistochemical staining in Ad-p202-treated tumors but not in tumors treated with Ad-Luc. Interestingly, strong p202 expression was found in endothelial cells of a tumor blood vessel. It may be due to systemic delivery of Ad-p202. As predicted, apoptosis, as determined by TUNEL assay, is prevalent in Ad-p202-treated tumors but not in Ad-Luc-treated tumors (Fig. 5*C*). The *arrows* indicate the representatives of apoptotic cells. This observation is consistent with our *in vitro* data showing that p202 expression induces apoptosis (Fig. 3). We have also performed a similar Ad-p202 preclinical gene therapy treatment in a human pancreatic cancer xenograft model (4). Consistent with the data presented here, Ad-p202 treatment (by i.t. injection) inhibited tumor growth and induced apoptosis in tumors (data not shown). Taken together, the above-mentioned observations strongly indicate that p202 is a potent tumor-suppressing agent, and its apoptosis-inducing activity contributes to the multiple p202-mediated antitumor activities.

DISCUSSION

In this report we showed that, consistent with our previous findings using p202 stable breast cancer cell lines (5), Ad-p202 infection in MDA-MB-468 breast cancer cells resulted in growth inhibition and sensitization to TNF-α-induced apoptosis. Importantly, we demonstrated for the first time that Ad-p202 infection alone induces apoptosis *in vitro*. The correlation between p202 expression and enhanced apoptosis observed in Ad-p202-treated tumors also supports the *in vitro* observation. However, it is possible that the apoptosis could be the result of an artifact caused by coexpression of p202 and adenoviral proteins. We ruled out that possibility because Ad-Luc infection of a p202 stable cell line, 453-p202 (5), did not result in enhanced apoptosis as compared with that of the vector control cells infected by Ad-Luc (data not shown). This result thus strongly suggests that the Ad-p202-induced apoptosis is not likely due to cooperation between p202 and certain adenoviral proteins during infection. Rather, it indicates that a certain cellular apoptotic pathway was activated by p202 expression. Indeed, as shown in Fig. 4, Ad-p202-induced apoptosis requires caspase-3 activation to achieve a full apoptotic effect.

Here, we demonstrated the feasibility of using Ad-p202 in preclinical gene therapy settings. In particular, Ad-p202 treatment by i.t. or i.v. injection resulted in significant tumor suppression in an orthotopic breast cancer xenograft model. Our data are consistent with that reported previously using p202 delivery systems other than adenoviral vector, *i.e.*, polymer and liposome (4, 5). The efficacy of systemic Ad-p202 treatment is encouraging because it shows that Ad-p202 had overcome immunological (nude mice possess immune response, albeit much reduced), physiological, and structural barriers inside and outside the blood vessels to reach tumor cells and unloads the p202 therapeutic gene (20). This result is the first demonstration of efficacy by systemic treatment of p202. It is possible that the systemic Ad-p202 treatment may affect normal tissues the same way it affects tumor tissues. One way to minimize the potential cytotoxicity of the p202 effect on normal tissues is to develop a tumor-specific p202 expression system using a breast cancer-specific promoter to direct p202 expression. This effort is currently in progress. Although toxicity, if any, associated with Ad-p202 treatment remains to be determined, our results nevertheless raise the possibility of using p202-based gene therapy in systemic cancer treatment. In Ad-p202-treated tumors, we also found a reduced level of an angiogenic marker, vascular endothelial growth factor (data not shown). This observation is consistent with the ability of p202 to inhibit angiogenesis (4).

In addition to prostate (data not shown) and breast cancer xenograft models (this study and Ref. 5), the fact that Ad-p202 treatment resulted in an antitumor effect on a pancreatic cancer xenograft model (data not shown; Ref. 4) suggests a general application of p202-based gene therapy in cancer treatment. In addition, because p202 sensitizes cells to TNF-α-induced apoptosis (this study and Ref. 5), our data further support the possible use of Ad-p202/TNF-α combined therapy to achieve better efficacy, especially for cancer cells that are resistant to TNF-α therapy. Experiments are under way to test this possibility in animal models. Taken together, the data we present here strongly suggest that Ad-p202 is a potent therapeutic agent suitable for further development in cancer gene therapy.

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p202, an interferon-inducible protein, inhibits E2F1-mediated apoptosis in prostate cancer cells

Duen-Hwa Yan,[a,b,c,*] Aram Abramian,[b] Zheng Li,[a] Yi Ding,[a] Yong Wen,[a,c] Ta-Jen Liu,[c,d] and Kelly Hunt[b,1]

[a] *Department of Molecular and Cellular Oncology, Box 79, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA*
[b] *Department of Surgical Oncology, Box 444, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA*
[c] *The University of Texas–Houston, Graduate School of Biomedical Sciences, Houston, TX 77030, USA*
[d] *Department of Neurooncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA*

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Abstract

p202, an interferon (IFN) inducible protein, is a phosphonuclear protein involved in the regulation of cell cycle, apoptosis, and differentiation. E2F1 belongs to the E2F family of proteins that are important cell cycle regulators in promoting cell growth. On the other hand, the deregulated expression of E2F1 also triggers apoptosis independent of p53 status. It has been well documented that p202 is able to inhibit cell growth by binding to E2F1 and abolishing the E2F1-mediated transcriptional activation of S-phase genes. However, it is not known whether E2F1-mediated apoptosis can be counteracted by p202 expression. Here, we show that E2F1-mediated apoptosis induced by the infection of an E2F1-expressing adenoviral vector (Ad-E2F1) was greatly diminished in p202-expressing prostate cancer cells. The E2F1-mediated caspase-3 activation was also reduced in p202-expressing cells infected with Ad-E2F1. Since caspase-3 is one of the E2F1 transcriptional targets, this result is consistent with the ability of p202 to inhibit the transcriptional activity of E2F1. Therefore, our results suggest a possible link between the IFN and E2F pathways in regulating apoptosis.

p202, a mouse interferon (IFN)-inducible protein, belongs to HIN-200 (hematopoietic interferon-inducible nuclear proteins with 200-amino-acid repeat) protein family in which the family members share a common structural feature of one or two 200 amino acid long homologous motifs [1–3]. The functional significance of p202 in cellular regulation is implicated by its physical interaction with several transcriptional factors that regulate cell cycle, including E2F1 [4]. These protein–protein interactions generally lead to transcriptional repression of promoters that are activated by these transcriptional factors.

E2F1 is a member of the E2F transcription factor family. Although E2F1 plays an important role during S-phase entry, it has been shown that overexpression of E2F1 is associated with induction of apoptosis [5–8]. p202 binds to the DNA binding domain of E2F1 and thus prevents E2F1 from binding to DNA. Consequently, this protein–protein interaction leads to transcriptional inhibition of many S-phase genes activated by E2F1, resulting in attenuation of S-phase entry [9]. However, it is not known if p202 can affect E2F1-mediated apoptosis. In this report, we demonstrate that while the infection of a recombinant adenoviral vector that expresses E2F1 (Ad-E2F1) readily induced apoptosis in a prostate cancer cell line, PC3, this E2F1-mediated apoptosis is significantly diminished in p202-expressing PC3 cells. Consistent with the above observation, we show that Ad-E2F1 infection correlates with the activation of an apoptotic molecule, caspase-3, that is inactivated in p202-expressing cells.

* Corresponding author. Fax: 1-713-794-0209.
E-mail address: dyan@mdanderson.org (D.-H. Yan).
[1] Also corresponding author. Fax: 1-713-792-4689.

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Materials and methods

Cell lines and adenovirus vectors. The human prostate cancer cell line, PC-3 [10], was cultured in DMEM/F12 media supplemented with 10% fetal bovine serum. The plasmids, pcDNA3 (Invitrogen, San Diego; CA) and CMV-p202 [9], were transfected into PC-3 cells. Four p202-expressing PC-3 stable clones (p202-1, -2, -3, and -4) were isolated [11]. The G418-resistant colonies from pcDNA3 transfection were pooled as a control. The construction of Ad-E2F1 has been described previously [6].

Western blot and apoptosis assays. Western blot analysis and PARP analysis were performed as described previously [6,12]. For flow cytometry analysis, cell lines were mock infected, or treated with Ad-Luc or Ad-E2F1 at an MOI of 100. Forty-eight hours following infection, cells were collected and then washed with PBS. Cell pellets were then fixed in 80% ethanol and kept a 4 °C until immediately prior to flow cytometric analysis. Cell pellets were then centrifuged, washed with PBS, and resuspended in propidium iodide solution (0.1% sodium citrate, 0.1% Triton X-100, 20 μg RNAse/ml, and 50 μg propidium iodide). Cells were then analyzed for DNA content (Epics Profile, Coulte Miami, Fla.).

Caspase-3 fluorometric assay. A caspase-3 fluorometric assay kit was utilized (R&D systems, Minneapolis, MN). Briefly, the Ad-E2F1, Ad-Luc (recombinant adenovirus expressing the luciferase reporter gene), and mock-infected cells were harvested at 48 h post-infection. Cell pellets were lysed with 25 μl/1 × 10^6 cells of cold lysis buffer (provided by the manufacturer). Fifty μl of cell lysate was then mixed with 50 μl of 2× reaction buffer (provided by the manufacturer) and 5 μl caspase-3 fluorogenic substrate (DEVD-AFC) in a 96-well plate. The reaction was incubated at 37 °C for 1 – 2 h followed by fluorescence analysis using a fluorescent microplate reader with light excitation at 400 nm and light emission at 505 nm.

Results and discussion

To test whether p202 affects the apoptosis induced by E2F1, we utilized Ad-E2F1 (MOI of 100) [6] or control adenovirus expressing the luciferase reporter gene (Ad-Luc) to infect four p202-expressing PC-3 stable cell lines (p202-1, -2, -3, and -4) [11] as well as the parental PC-3 and the pooled empty-vector (pcDNA3) transfected PC-3 cells. To confirm the expression of E2F1 in Ad-E2F1-infected cells, we performed a Western blot to detect E2F1 expression using an E2F1-specific antibody. As shown in Fig. 1, E2F1 is readily detectable in Ad-E2F1-infected cells (E) but not in the mock (C)- or Ad-Luc (L)-infected cells. To examine the effect of p202 expression on E2F1-mediated apoptosis, we subjected the virus (Ad-E2F1 or Ad-Luc)-infected cells to flow cytometry analysis in order to assess the percentage of sub-G1 (apoptotic) cell population. The percentage of apoptosis in the Ad-E2F1-infected cells was normalized to the Ad-Luc-treated cells, i.e., percentage of sub-G1 of Ad-E2F1 infection/percentage of sub-G1 of Ad-Luc infection (Fig. 2A). While it is expected that Ad-E2F1 induced apoptosis in PC-3 and pcDNA3 vector-transfected control cells, the E2F1-induced apoptosis is greatly attenuated in all p202-expressing cells. To further confirm this observation, we employed another apoptosis assay, i.e., poly(ADP-ribose) polymerase (PARP) cleavage assay, in which intact 116 kDa PARP is cleaved by caspases into a fragment of approximately 85 kDa [12]. The cell lysate isolated from Ad-Luc (L) and Ad-E2F1 (E) treated cells was analyzed by Western blot using PARP-specific antibody. As shown in Fig. 2B, while Ad-E2F1 infection resulted in PARP cleavage indicative of apoptosis in PC-3 and pcDNA3 vector-transfected cells, the E2F1-induced PARP cleavage was significantly inhibited in p202-2 (a p202-expressing cell line that has relatively higher p202 expression than the other p202 stable cell lines [11]). Taken together, these results strongly indicate that p202 is able to inhibit apoptosis induced by overexpression of E2F1. Since a variety of substrates including PARP [13] are cleaved during the cascade of caspase activation leading to apoptosis, our results suggest that the activation of caspases may be involved in Ad-E2F1-induced apoptosis. Furthermore, recent report suggests that the activation of caspase-3 is, at least in part, responsible for the E2F1-mediated apoptosis [14]. To test whether the inhibition of E2F1-mediated apoptosis in p202-expressing cells correlates with the inhibition of caspase-3 activity, we performed the caspase-3 fluorometric assay. Briefly, the cell lysates isolated from Ad-E2F1 and Ad-Luc infected cells as well as the untreated cells were incubated with caspase-3-specific fluorogenic substrate (DEVD-AFC) followed by fluorescence analysis at 505 nm. The intensity of fluorescence is indicative of caspase-3 activity. As shown in Fig. 3, Ad-E2F1 infected PC-3 and pcDNA3 vector-transfected cells exhibited higher caspase-3

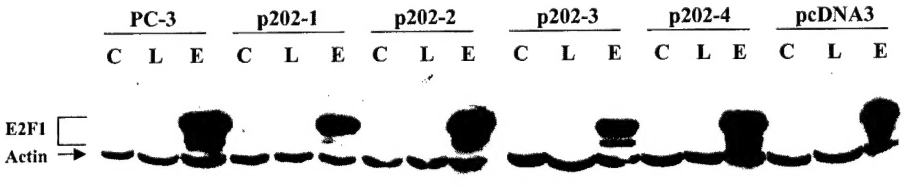

Fig. 1. E2F1 overexpression by Ad-E2F1 in human prostate cancer cells. Parental PC3 cells, pcDNA3 vector-transfected cells, and p202-expressing cells (p202-1, p202-2, p202-3, and p202-4) were mock infected (C), or infected with Ad-Luc (L) or Ad-E2F1 (E) at an MOI of 100. Forty-eight hours following infection, cell lysates were prepared and 50 μg of protein was subjected to Western blot analysis. Nylon membranes were probed with antibodies to E2F1 and actin. Actin expression was used to confirm equal protein loading.

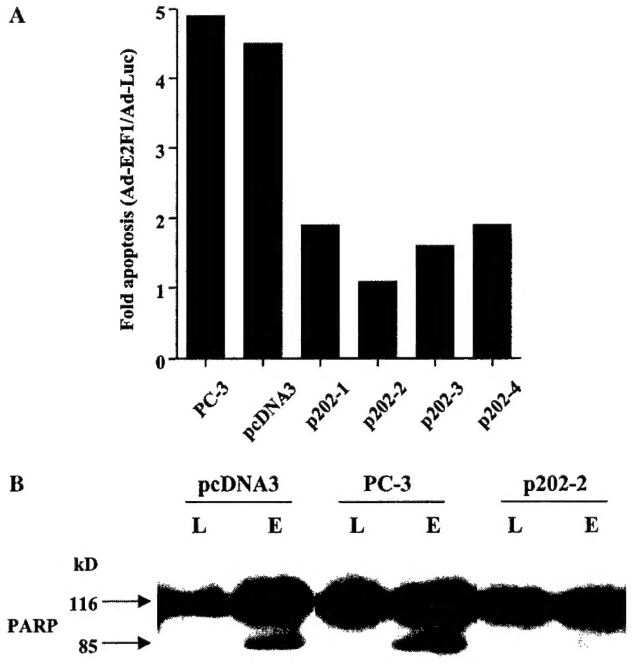

Fig. 2. Attenuation of E2F1-mediated apoptosis in p202-expressing prostate cancer cells. (A) Apoptosis determined by flow cytometry assay. Cell lines were mock infected, or treated with Ad-Luc or Ad-E2F1 at an MOI of 100. Forty-eight hours following infection, cells were harvested and subsequently analyzed by flow cytometry (Materials and methods). The subdiploid population was calculated and recorded as percentage of apoptotic cells. (B) PARP cleavage was asessed by Western blot analysis. Protein was collected from cells following treatment with Ad-E2F1 (E) or Ad-Luc (L). Protein extracts were subjected to gel electrophoresis and then transferred to a nylon membrane. Membranes were probed with an antibody against PARP. The arrows indicate the full-length PARP (116 kDa) and the cleaved fragment (85 kDa).

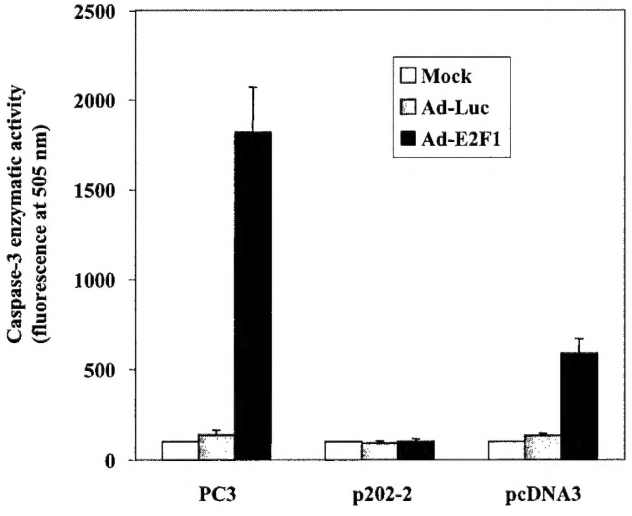

Fig. 3. The level of caspase-3 activity is measured by caspase-3 fluorometric assay. The cells were infected by Ad-Luc or Ad-E2F1 for 48 h. Untreated cells were used as control. The cell lysates were then tested for caspase-3 activity by the addition of a caspase-specific peptide that is conjugated to the fluorescent reporter AFC. The fluorescence signals were detected at 505 nm after being excited by light at 400 nm wavelength.

activity than the controls (mock infection or Ad-Luc infection). In contrast, the caspase-3 activity of the Ad-E2F1-infected cells expressing p202 was at the basal level of those infected with Ad-Luc or without treatment. This result suggests that caspase-3 inactivation is involved in the inhibition of E2F1-mediated apoptosis by p202.

It has been shown that p202-mediated growth retardation is associated with the inactivation of E2F1 via p202/E2F1 interaction [9]. In this report, we show that p202 is able to inhibit E2F1-mediated apoptosis and that this is associated with the inactivation of caspase-3. Given the observation that E2F1 induces caspase-3 expression, it is likely that p202 inhibits the E2F1-mediated apoptosis by abolishing the transcriptional activity of E2F1 and that in turn represses caspase-3 expression, leading to attenuation of apoptosis. Several lines of evidence seem to suggest that the interacting protein partners determine the functional role of p202 in apoptosis. For instance, p202 binds c-Myc and inhibits c-Myc-mediated apoptosis [15]. p202 could also attenuate the apoptosis induced by p53, presumably by inactivating the p53 transcriptional activity via p202/p53 interaction (K. Hunt, unpublished results, [16,17]). In contrast, we have shown that p202 interacts with the anti-apoptotic molecule, NF-κB, and sensitizes cells to apoptosis induced by TNF-α [12,18]. Together, these observations demonstrate the versatility of p202 in controlling the cell fate by interacting with different cell cycle regulators or survival factors in response to different extracellular stimuli such as growth factor or cytokines. Therefore, our results presented here suggest a possible cross talk between the IFN and E2F pathways in regulating apoptosis.

Acknowledgments

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